DETERMINATION OF ANTIBIOTIC RESISTANCE IN SELECTED CLINICAL BACTERIAL ISOLATES

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1. INTRODUCTION

1.1. Historical overview of antibiotics

The antibiotics field was initiated when Paul Ehrlich first coined the term ‘magic bullet’, or chemotherapy, to designate the use of antimicrobial compounds to treat microbial infections. In 1910, Ehrlich discovered the first antibiotic drug, Salvarsan, which was used against syphilis. Ehrlich was followed by Alexander Fleming, who discovered penicillin by accident in1928. Then, in the 1935, Gerhard Domagk discovered the sulfa drugs, thereby paving the way to the discovery of the anti-TB drug Isoniazid. Then, in 1939 Rene Dobos became then first scientist to discover an antibiotic after purposely looking for it in soil microbes. Dubos discovered Gramicidin, which is still used today to treat skin infections. Finally, in 1943, the first TB drug, Streptomycin, was discovered by Selman Waksman and Albert schatz. Waksman was also the one who coined the term ‘antibiotics’. Thus, antibiotics have been used to treat bacterial infections since the 1940(Ying Zhang Baltimore2007,USA))

1.2 Antibiotic

An antibiotic is an agent produced by a microbe, such as a fungus or a bacterium, that kills or slows the growth of other microorganisms, particularly bacteria. An antibiotic is a type of antimicrobial drug. Antibiotics are used to treat bacterial illnesses like ear infections and strep throat. Antibiotics and antimicrobial drugs are also used to treat or prevent illnesses in animals and in other areas of agriculture. Antimicrobial agents are also found in many of the cleaning products marketed today. The use of antimicrobial agents can lead to the development of antimicrobial resistance in microbes exposed to them. (Richard C. Honour 2003)

1.3. The Basic Characteristics of Antibiotics

Today, there are about 4000 compounds with antibiotic properties. Antibiotics are used to treat and prevent infections, and to promote growth in animals. Antibiotics are derived from three sources: moulds or fungi; bacteria; or synthetic or semi-synthetic compounds. They can be used either internally or topically, and their function is to either inhibit the growth of pathogens or to kill them. Antibiotics can thus be divided into Bacteriostatic drugs, which merely inhibit the growth of the

pathogen, and Bacteriocidal drugs, which actually kill the bacteria. However, the distinction is not absolute, and depends on the drug concentration, the bacterial species, and the phase of growth. Antibiotics are more effective against actively growing bacteria, than against non-growing per sisters or spores. When two antibiotics are used in combination, the effect could be additive, synergistic, or antagonistic. Antibiotics can also be divided into broad-spectrum and narrow-spectrum antibiotics. For example, Tetracycline, a broad spectrum antibiotic, is active against G+ bacteria, G bacteria, and even against mycobacteria; whereas penicillin, which has a relatively narrow spectrum, can be used mainly against G+ bacteria. Other antibiotics, such as Pyrazinamide, have an even narrower spectrum, and can be used merely against Mycobacterium tuberculosis.Antibiotics fight against bacteria by inhibiting certain vital processes of bacterial cells or metabolism. Based on these processes, we can divide antibiotics into five major classes:

1. Cell wall inhibitors, such as Penicillin and Vancomycin.
2. Inhibitors of nucleic acid synthesis, such as Fluoroquinolones, which inhibits DNA synthesis, and rifampin, which inhibits RNA synthesis.
3. Protein synthesis inhibitors, such as Amino glycoside.
4. Anti-metabolites, such as sulfa drugs.
5. Antibiotics that can damage the membrane of the cell, such as Polymyxin B, Gramicidin and Daptomyc in (YingZhang Baltimore2007,
   USA)

1.4. The Origin of Antibiotic Resistance

Although it is difficult to know for certain, the absolute original source of antibiotic resistance may be the bacteria themselves that produced the antibiotics in the first place. Within the microbial world there is the concept of microbial antagonism, where by each kind or species of bacteria is in direct competition for nutrients and space with other microbes. Somewhere in the process, bacteria developed chemical defenses against their competitors, and today we refer to those chemicals as antibiotics. (Richard C. Honour 2003)

Nearly all microbes, and even higher organisms, such as algae, fungi and higher plants, all make chemicals to overcome or otherwise control the competition. It was the early observations of ‘zones of inhibition’ in the growth patterns of bacteria

on the surfaces of Petri plates caused by moulds that provided the observations. And historically, organic soils, peat, humus, mosses and moulds of many kinds were used in natural and historical medicine to treat infections. Those organic materials used in traditional medicine all harbored microbes that produced chemical antibiotics. (Richard C. Honour 2003)

1.5. Types of antibiotic resistance

There are three types of resistance: natural (intrinsic or innate), acquired and mutational.(Ashraf,R and Shah,N,P 2011)

1.6. The Rise of Antibiotic-Resistant Infections

When penicillin became widely available during the second world war, it was a medical miracle, rapidly vanquishing the biggest wartime killer--infected wounds. Discovered initially by a French medical student, Ernest Duchesne, in 1896, and then re discovered by Scottish physician Alexander Fleming in 1928, the product of the soil mold Penicillium crippled many types of disease-causing bacteria. But just four years after drug companies began mass-producing Penicillin in 1943, microbes began appearing that could resist it. (Ricki Lewis 1995)

The first bug to battle penicillin was *Staphylococcus aureus*. This bacterium is often a harmless passenger in the human body, but it can cause illness, such as pneumonia or toxic shock syndrome, when it overgrows or produces a toxin.

In 1967, another type of penicillin-resistant pneumonia, caused by *Streptococcus pneumoniae* and called Pneumococcus, surfaced in a remote village in Papua New Guinea. At about the same time, American military personnel in Southeast Asia were acquiring penicillin-resistant Gonorrhea from prostitutes. By 1976, when the soldiers had come home, they brought the new strain of Gonorrhea with them, and physicians had to find new drugs to treat it. In 1983, a hospital-acquired intestinal infection caused by the bacterium *Enterococcus faecium* joined the list of bugs that outwit penicillin.

Antibiotic resistance spreads fast. Between 1979 and 1987, for example, only 0.02 percent of Pneumococcus strains infecting a large number of patients surveyed by the National Centers for Disease Control and Prevention (CDC) were Penicillin-

resistant. CDC's survey included 13 hospitals in 12 states. Today, 6.6 percent of pneumococcus strains are resistant, according to a report in the June 15, 1994, Journal of the American Medical Association by Robert F.Breiman, M.D., and colleagues at CDC. The agency also reports that in 1992, 13,300 hospital patient died of bacterial infection that were resistant to antibiotic treatment.(Ricki Lewis 1995)

1.7. Antibiotic Resistance or Antibiotic resistance by microbes

Antibiotic resistance has developed as a natural evolutionary response of bacteria following their exposure to these drugs and is defined as the ability of bacteria to withstand the inhibition concentration of an antibiotic. It can be intrinsic (naturally occurring) or acquired. The first type is defined as resistance characteristic for all members of a given bacterial species or genus. It results from an inability of the antibiotic to reach its target, a lack of affinity for the target, the presence of efflux pumps or possession of other chromosomal resistance mechanisms (Magdalena Chroma 2010)

Antibiotic resistance is recognized as being one of measures to prevent, or contain, the increase in antibiotic resistant organisms requires knowledge of both the genesis of an antibiotic resistance genes and their dissemination. The problem of antibiotic resistance affects almost every bacterial species for which treatment with antibiotics is available (Adel Kk and Sabiha,S.S 2010)

Resistant bacteria are not as susceptible to antibiotics as non-resistant bacteria. The use of antibiotics may eliminate susceptible bacteria, leaving resistant bacteria behind. If resistant bacteria spread, a person or animal with this infection may not be able to be treated with the usual antibiotics, or an increased dose may be required. As a result, they may be sick for a longer time than if they had an infection caused by bacteria that were easily treatable with antibiotics. (Richard C. Honour 2003)

1.8. Development of Resistance

The increase in bacterial antimicrobial resistance is a natural phenomenon, an outcome of evolution. Any population of organisms, including bacteria, naturally includes variants with unusual traits. In this case, some bacteria have the ability to fend off the action of an antimicrobial agent. The use of antimicrobial drugs in

humans and animals over the past 50 years has inadvertently accelerated the development of resistance by increasing the selective pressure exerted on these organisms. Once antimicrobial pressure has been introduced into an environment, resistance may be spread to other microbes. Food animals, such as cattle, pigs, turkeys or chikens, may receive antimicrobial drugs for growth promotion and control or treatment of infectious diseases.

Food animals can carry organisms that can make people sick, but may not necessarily make the animal sick. For example, *Salmonella, Campylobacter and E. coli* are common bacteria found in the intestines of various food animals. These bacteria may not cause disease in the animal, but all three bacteria may cause foodborne illness in humans. These organisms may develop resistance when exposed to antibiotics given to the animal. These resistant organisms can contaminate food products at slaughter and then infect humans who eat the food, particularly if the food is undercooked or contaminated after cooking. Evidence of increasing resistance to antimicrobial drug treatment in bacteria that infect humans has raised questions about the role that antimicrobial drug use in food animals plays in the emergence of antimicrobial drug resistant bacteria. The link between antimicrobial resistance in foodborne pathogenic bacteria and use of antimicrobials in food animals has been reported in a number of studies. For foodborne pathogens, especially those such as Salmonella that are rarely transferred from person to person in the US, food (such as meat or eggs) from food animals is considered a likely source of most antimicrobial resistance (Richard C. Honour2003)

1.9. Causes for Drug Resistance

One of the main causes of antibiotics drug resistance is antibiotic overuse, abuse, and in some cases, misuse, due to incorrect diagnosis. A second cause is counterfeit drugs. Antibiotic use Antibiotic use in animal husbandry is also creating some drug resistant bacteria, which can be transmitted to humans. Increased globalisation could also cause the spread of drug resistance. Finally, hospital settings often give rise to antibiotic resistant bacteria.(Ying ZHANG Baltimore, 2007.USA)

1.10. Prevent the Spread of Antimicrobial Resistance

- Unless otherwise bacterial persist Antibiotic treatment may be avoided to relieve the symptoms
- Exact medication given by prescription need to be taken
- Entire course of antibiotic must be taking even if relief from symptoms.
- Repetition of same medication need to be avoided (Richard C. Honour 2003)

1.11. Possible mechanisms involved in antibiotic resistance

Bacteria may manifest resistance to antibacterial drugs through a variety of mechanisms. Some species of bacteria are innately resistant to 1st class of antimicrobial agents. In such cases, all strains of that bacterial species are likewise resistant to all the members of those antibacterial classes of greater concern are cases of acquired resistance, where initially susceptible populations of bacteria become resistant to an antibacterial agent and proliferate and spread under the selective pressure of use of that agent. (yingzhang 2007) Several mechanisms of antimicrobial resistance are readily spread to a variety of bacterial genera. There are five major mechanisms of antibiotic drug resistance, which are due to chromosomal mutations:

- Reduced permeability or uptake.
- Efflux pump
- Enzymatic inactivation.
- Alteration of the drug target.
- Loss of enzyme in drug activation (Magdalena Chroma 2010. Ying Zhang Baltimore 2007, USA).

a. Reduced Permeability or Uptake

The first mechanism is reduced permeability or uptake of the bacteria. For example, *Neisseria gonorrhoea* porin can acquire mutations that can cause resistance to penicillin and tetracycline. Another example is *Enterobacter aerogenes* porin, which can acquire mutations that cause cephalosporin resistance.

b. Increased Efflux Activity

Efflux pumps are transmembrane transport proteins, used physiologically in Gram positive and Gram-negative bacteria for exporting specific metabolites and xenobiotic toxic substances out of the cell. As an energy source they utilize the proton

motive force. Pumps may be specific for one substrate or may transport arrange of trructurally different compounds (including multiple antibiotics); such transport proteins can be associated with multiple drug resistance. Tetracycline pumps are probably the best studied efflux system in both Gram-positive and Gramnegative bacteria. (Magdalena Chroma *et al* 2010)

There are many examples of the second mechanism, increased efflux activity. The first one, Tetracycline efflux was discovered in the early 1980s. TetK serves as an example for an efflux-mediated Tetracycline resistance. Under normal conditions, the efflux gene, TetK, is not expressed, due to a suppressor that is bound to the promoter region. However, in the presence of Tetracycline, it binds to the repressor, relieves the suppression, and causes transcription and translation of the efflux pump, thereby leading to Tetracycline resistance.(Ying Zhang Baltimore 2007).

c. Enzymatic Inactivation

A famous example of the third mechanism, enzymatic inactivation, is Beta-lactamases, which can cleave beta-lactam antibiotics and cause resistance. A second example is the Aminoglycoside-inactivating enzymes, which can add Acetyl, Adenyl, and Phosphoryl groups to inactivate the antibiotic. Finally, both Chloramphenicol Acetyl Transferase and Streptogramin Acetyl Transferase can add an acetyl group to inactivate the two antibiotics, respectively.

d. Alteration of Drug Target

An example close to home to the alteration or over-expression of the drug target is InhA. This promoter mutation causes over-expression of the drug target InhA, and lead to a low-level isoniazid (INH) resistance in *M. tuberculosis*. A second noteworthy example is penicillin resistance, which is due to alterations in penicillin binding proteins. A third example is vancomycin resistance. Under susceptible conditions, vancomycin prevents cross-linking of peptidoglycan by binding to D-Ala-D-Ala dipeptide of the muramylpeptide. Most G+ bacteria acquire vancomycin resistance by changing D-Ala-D-Ala to DAla-D-lactate, which does not bind to vancomycin. A fourth example is mutations in DNA gyrase A and B subunits in quinolone resistance is another example of an alteration of the drug target. Finally, in

Rifampicin resistance, there are mutations in *rpoB* gene encoding beta-subunit of RNA polymerase.

e. Loss of Enzymes in Drug Activation

Loss of enzymes involved in drug activation is a relatively new mechanism of drug resistance. In this case, the antibiotic itself is a prodrug, which has no direct activity against the bacteria. Rather, it relies on the activation by a bacterial enzyme.INH can serve as a useful example. KatG (catalase-peroxidase) is an enzyme involved in the activation of INH, which produces a range of reactive metabolites including reactive oxygen species and then reactive organic radicals, which then inhibit multiple targets, including mycolic acid synthesis. Another example is the Metronidazole (MTZ) prodrug. MTZ is activated through RdxA (nitroreductase), and then forms reactive species that damage the DNA. Thus, mutations in this enzyme cause resistance to Metronidazole.

1.12. Experimental studies supporting determination of bacterial resistance

1.12.1. Minimum inhibitory concentration

MIC was defined as the lowest concentration of antibiotics that produced no growth Minimum Inhibitory Concentration (MIC) (lowest concentration of an antimicrobial agent that inhibits growth of microorganism after overnight incubation (Amin *etal* 2009)

1.12.2. Transformation

One bacterium may take up DNA from another, either as a single gene or more often as cassettes of genes, which are then spliced into the chromosome of the new host bacterium. Bacteriophage viruses also transfer DNA from one bacterium to another, sometimes among bacteria of the same species and sometimes across Genera. (Richard C. Honour2003)

1.12.3. Plasmid DNA

Small circular forms of DNA called plasmids. Plasmids carrying resistance genes may be exchanged between bacteria of different species or types. (Richard C. Honour2003)

Plasmids are most replicating circular pieces of DNA, smaller than the bacterial genome which encoded their transfer by replication into another bacterial strain or species. They can carry and transfer multiple resistance genes, which may be located on section of DNA capable of transfer from one plasmid to another or to the genome-transposon or jumping gene (Adel Kk and Sabiha,S.S 2010)

1.12.4. Competence cell

Natural competence is a genetically programmed physiological state permitting the efficient uptake of macromolecular DNA. It is distinct from artificial transformation involving electroporation, protoplasts, and heat shock/CaCl2 treatment. In many bacteria, competence is highly regulated, and much research has been devoted to exploring the complex control mechanisms involved. (David Dubnau 1999)

1.12.5. Efflux pump

Efflux pumps are transports proteins involved in the extrutions of toxic substrate (including virtually all classes of relevant antibiotics) from within the cells into the external environment.these proteins are found in both Gram-positive and Gram-negative bacteria as well as in eukaryotic organism. Pump may be specific for one substrate or transport a range of structurally dissimilar compounds (including antibiotic of multiple classes), such pump can be associate with multiple drug resistance MDR. In the prokaryotic kingdom there are five major families of efflux transporters MF (Major facilitator),MATE (mutidrug and toxic efflux), RND (resistance –nodulation-division), SMR (small mutidrug resistance), and ABC (ATP binding cassette). All these system utilize the proton motive force as an energy, apart from the ABC family, which utilize ATP hydrolysis to drive the export of substrates. (Webber M.A. and Piddock L.J.V. 2003)

1.12.6. Necessity to determine the resistance

- To prevent increase in antibiotic resistance,
- To prevent/contain spread of infection,

- To find out alternative drug by understanding the Mechanisms. So the current study focused to necessity to determination resistance bacteria, and its mechanism. The scope existent in development of new antibiotic and prevention of spread specific resistance. (adel k.k *et al* 2010)

2. LITERATURE REVIEW

Lazaroaie MM 2009 stated that the high world interest given to the researches concerning the study of moderately halophilic solvent-tolerant bacteria isolated from marine polluted environments is due to their high biotechnological potential, and also to the perspective of their application in different remediation technologies. Using enrichment procedures, two moderately halophilic Gram-negative bacterial strains were isolated from seawater sample, which are tolerant to organic solvents. From their experience they stated that the cell tolerance, adhesion and cells viability of *Aeromonas salmonicida* IBBCt2 and *Pseudomonas aeruginosa* IBBCt3 in the presence of organic solvents depends not only on its physicochemical properties and its concentration, but also on the specific response of the cells, and the cellular response is not the same for those bacterial strains. n-hexane, n-heptane, propylbenzene, with log POW between 3.69 and 4.39, were less toxic for *Aeromonas salmonicida* IBBCt2 and *Pseudomonas aeruginosa* IBBCt3, compared with toluene, styrene, xylene isomers and ethylbenzene, with log POW between 2.64 and 3.17. The results indicated that *Aeromonas salmonicida* IBBCt2 is more susceptible to organic solvents than *Pseudomonas aeruginosa* IBBCt3. The mechanisms underlying solvent tolerance (e.g., the existence of the efflux pumps) in *Aeromonas salmonicida* IBBCt2 and *Pseudomonas aeruginosa* IBBCt3 it was also studied.

Edward IA *et al* 2012 studied the petroleum contaminated site has a high concentration of organic solvents and heavy metals. Bacteria surviving in the contaminated site will implement efflux systems to evade from toxic organic solvents. In this study, an attempt was taken to perceive the relation between efflux pump activity and organic solvent tolerance of bacteria. B. oleronius isolated from petroleum contaminated site was utilized to investigate the role of efflux pump in organic solvent tolerance. The viability of cells was significantly reduced in broth supplemented with test solvents compared to untreated bacteria. Rhodamine B accumulation and efflux studies divulged that the bacterial isolate has greatly utilized

the efflux pump system to accumulate and siphon out the dye. The intrinsic correlation between solvent tolerance and efflux pumps was studied by treating the bacterial isolates with efflux pump inhibitors, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and 2,4 Dintrophenol (DNP) in the presence and absence of organic solvents. Compared to the control, treated isolates have failed to grow and showed different growth pattern indicating the critical role of efflux pumps in solvent tolerance. Antimicrobials potentiation of solvent tolerance on B. oleronius has showed significant changes in the growth pattern and presaged the activation of efflux pump which in turn activated the solvent tolerance mechanisms. Their results showed that bacteria employ a wide array of efflux systems to tackle the high concentration of solvents in the contaminated site.

Neetu Kumra Taneja *et al* 2007 developed simple, rapid, low-cost and robust assays for screening drugs against dormant and actively growing mycobacteria. Actively growing aerobic and hypoxia-adapted dormant cultures of Mycobacterium tuberculosis, Mycobacterium bovis BCG and Mycobacterium smegmatis were tested for susceptibility to standard antimicrobial drugs by resazurin reduction assay. The visual and fluorimetric MICs were compared with those obtained by the standard cfu assay. Drug MICs for M. tuberculosis and M. bovis BCG were determined by the aerobic resazurin microplate assay (REMA) and correlated well with those obtained by the cfu assay. Metronidazole and nitrofurans showed comparable bactericidal activity in the hypoxic resazurin reduction assay (HyRRA). The HyRRA assay was noted to be superior to the cfu assay in that it distinguished between metabolically active dormant bacteria and non-viable organisms, unlike the cfu assay that could not differentiate between these two populations. The HyRRA assay performed with good concordance in both fluorimetric and visual formats to distinguish between bactericidal and bacteriostatic effects of a drug. They suggested that REMA and HyRRA assays will be useful for anti-tubercular anti-dormancy compound screening and drug susceptibility testing in a safe, reliable, easy and cost-effective manner particularly in low resource countries. The application of the assays in M. smegmatis or M. bovis BCG offers the distinct advantage of rapidly and safely screening anti-tubercular compounds in a high-throughput format.

Ashraf R and Shah NP2011 reported that intrinsic resistances to tetracycline, vancomycin and erythromycin are common in Lactobacillus species; however, resistance to streptomycin, clindamycin, gentamicin, oxacillin and lincosamide were also reported in those species. Resistant markers tet(W), tet(M) and erm(B) have been frequently detected in the resistant strains while van(A), lnu(A) and tet(L) have also been found in some strains of Lactobacillus. Bifidobacteria are commonly resistant to tetracycline, streptomycin, erythromycin, gentamicin and clindamycin. Resistance genes van(A), tet(L) and tet(M) are often detected in *Enterococcus*. Reports suggest *enterococci* to transfer tet(M) to *E. faecalis or Listeria* strains and van(A) to commercial strain of Lactobacillus acidophilus. *Streptococcus* species are highly resistant to tetracycline, ciprofloxacin and aztreonam and tet(M) was detected in strains of dairy origin. Clinical cases of *endocarditis, septicemia, bacteremia* and septic arthritis due to the species of *Lactobacillus, Saccharomyces, Leuconostoc, Pediococcus and Bifidobacterium* have been reported in patients with some underlying medical conditions.

Nizami Duran *et al* 2012 evaluated the association between the antibiotic susceptability patterns and the antibiotic resistance genes in staphylococcal isolates obtained from various clinical samples of patients attending a teaching hospital in Hatay, Turkey.

Mradula Singh *et al* 2009 studied the drug resistant, especially multi-drug resistant (MDR) tuberculosis, fluoroquinolones (FQs) are used as second line drugs. However, the incidence of FQ-resistant Mycobacterium tuberculosis is rapidly increasing which may be due to extensive use of FQs in the treatment of various other diseases. The most important known mechanism i.e., gyrA mutation in FQ resistance is not observed in a significant proportion of FQ resistant M. tuberculosis isolates suggesting that the resistance may be because of other mechanisms such as an active drug efflux pump. In this study we evaluated the role of the efflux pumps in quinolone resistance by using various inhibitors such as carbonyl cyanide m-chlorophenyl hydrazone (CCCP), 2, 4-dinitrophenol (DNP) and verapamil, in clinical isolates of M. tuberculosis

Janne Kataja and Pentti Huovinen 1998 studied *Streptococcus pyogenes* isolates (group A streptococcus) of different erythromycin resistance phenotypes

were collected from all over Finland in 1994 and 1995 and studied; they were evaluated for their susceptibilities to 14 antimicrobial agents (396 isolates) and the presence of different erythromycin resistance genes (45 isolates). The erythromycin-resistant isolates with the macrolide-resistant but lincosamide- and streptogramin Bsusceptible phenotype (M phenotype) were further studied for their plasmid contents and the transferability of resistance genes. Resistance to antimicrobial agents other than macrolides, clindamycin, tetracycline, and chloramphenicol was not found. When compared to our previous study performed in 1990, the rate of resistance to tetracycline increased from 10 to 93% among isolates with the inducible resistance (IR) phenotype of macrolide, lincosamide, and streptogramin B (MLSB) resistance. Tetracycline resistance was also found among 75% of the MLSB-resistant isolates with the constitutive resistance (CR) phenotype. Resistance to chloramphenicol was found for the first time in *S. pyogenes* in Finland; 3% of the isolates with the IR phenotype were resistant. All the chloramphenicol-resistant isolates were also resistant to tetracycline. Detection of erythromycin resistance genes by PCR indicated that, with the exception of one isolate with the CR phenotype, all M-phenotype isolates had the macrolide efflux (mefA) gene and all the MLSB-resistant isolates had the erythromycin resistance methylase (ermTR) gene; the isolate with the CR phenotype contained the ermB gene. No plasmid DNA could be isolated from the M-phenotype isolates, but the mefA gene was transferred by conjugation.

Alasdair P MacGowan *et al* 2003 carried out the antibacterial effect and emergence of resistance to gemifloxacin and levofloxacin in an in vitro pharmacokinetic model of infection. A panel of Streptococcus pneumoniae strains with known mechanisms of resistance were used; two strains had no known resistance mechanism, two had efflux pumps, three had gyrA plus parC mutations, and one had only a parC mutation. Gemifloxacin MICs were in the range of 0.016 to 0.25 mg/liter, and levofloxacin MICs ranged from 1 to 16 mg/liter. Antimicrobial effect was measured by area under the bacterial-kill curve up to 72 h, and emergence of resistance was determined by population analysis profile before and during drug exposure. The area under the curve (AUC)/MIC ratios for gemifloxacin and levofloxacin were 35 to 544 and 3 to 48, respectively. As expected on the basis of these AUC/MIC ratio differences, antibacterial effect was much greater for gemifloxacin than levofloxacin. In the gemifloxacin simulations, mechanism of

resistance as well as MIC determined the antibacterial effect, as indicated by gemifloxacin's greater effect against efflux strains compared to those with gyrA or parC mutations despite similar MICs. This was not true of levofloxacin. Emergence of resistance was not easily demonstrated with either agent, and mechanism of resistance did not have any impact on it.

Thomas Schwartz 2002 viewed of the increasing interest in the possible role played by hospital and municipal wastewate systems in the selection of antibioticresistant bacteria, biofilms were investigated using *enterococci, staphylococci*, *Enterobacteriaceae,* and heterotrophic bacteria as indicator organisms. In addition to wastewater, biofilms were also investigated in drinking water from river bank filtrate to estimate the occurrence of resistant bacteria and their resistance genes, thus indicating possible transfer from wastewater and surface water to the drinking water distribution network. Vancomycin-resistant *enterococci* were characterized by antibiograms, and the vanA resistance gene was detected by molecular biology methods, including PCR. The vanA gene was found not only in wastewater biofilms but also in drinking water biofilms in the absence of enterococci, indicating possible gene transfer to autochthonous drinking water bacteria. The mecA gene encoding methicillin resistance in staphylococci was detected in hospital wastewater biofilms but not in any other compartment. *Enterobacterial* ampC resistance genes encoding L-lactamase activities were amplified by PCR from wastewater, surface water and drinking water biofilms

Gary S Gray1980 sequenced kanamycin resistance gene from *Staphylococcus aureus* and its structure was compared with similar genes isolated from *Streptomycesfradiae* and from two transposons, Tn5 and Tn903, originally isolated from *Klebsiella pneumoniae* and *Salmonella typhirnurium*, respectively. The genes are all homologous but, since their common ancestor, have undergone extensive divergence, with more than 43% divergence between the closest pair. The phylogeny of the genes cannot be made congruent to the phylogeny of the taxa from which they were isolated without requiring rather improbable differences in rates. One is therefore led to conclude that there have been multiple occurrences of gene transfer between these species. Thus, although they are homologous, they are neither

orthologous nor paralogous. It was suggested that homologous genes of this type be called xenologous.

Roberto Cabrera and Joaquím Ruiz 2004 studied the evolution of antimicrobial resistance in *Salmonella* isolates causing traveler's diarrhea (TD) and their mechanisms of resistance to several antimicrobial agents were analyzed.

Anandi Martin *et al* 2003 worked on 150 *Mycobacterium tuberculosis* isolates against the second-line drugs ethionamide, kanamycin, capreomycin,ofloxacin, and para-aminosalicylic acid by the colorimetric resazurin microtiter assay and the proportionmethod. By visual reading, MICs were determined after 8 days and a very good correlation between results by the colorimetric resazurin microtiter assay and the proportion method were reported. The colorimetric resazurin microtiter assay is inexpensive, rapid, and simple to perform, and implementation of the assay is feasible for low-resource countries.

Satyajit D. Sarker *et al* 2007 modified the resazurin assay utilising microtitre-plate, described by Drummond and Waigh in 2000, to achieve more accuracy in the determination of the minimum inhibitory concentration (MIC) values of natural products, including crude extracts, chromatographic fractions or purified compounds against various bacterial strains. This modified resazurin method is simple, sensitive, rapid, robust and reliable, and could be used successfully to assess antibacterial properties of natural products.

Adel KK and Sabiha SS 2010 determined the site of antibiotic resistant genes in *Pseudomonas aeruginosa. 50 P Aeruginosa* were isolated out of 250 samples from hospitalized patients in different hospitals in Sulaymani city, Iraq, more over isolated from soil dustbin and sewage water. The isolates were identified according to the cultural, morphological and biochemical tests, in addition to API 20E system. The isolated bacteria were screened for their resistance to seventeen commonly used antibiotics. The isolated *P. aeruginosa* show different pattern of antibiotic resistance, they grouped in to 34 antibiotype. Organisms were 100% resist to (Amp, Cef, Er, Gm, Lin, Pip and Cln), the lowest resistant recorded was 35% for Rif. Six *P. aeruginosa* isolates 12% revealed resistant to all seventeen used antibiotics, while nine isolates 18% were sensitive to one antibiotic, and 10 isolates were sensitive to two antibiotics,

and remaining 50 isolates were sensitive to 3-5 antibiotics. Transformation process appeared that the genes responsible for all tested antibiotics except Lin in P1 isolate, and Lin and Tob in P8 isolate are located on plasmid DNA in isolated *P. aeruginosa.*

Kunihiko Nishino *et al* 2003 stated that histone-like protein H-NS is a major component of the bacterial nucleoid and plays a crucial role in global gene regulation of enteric bacteria. It is known that the expression of a variety of genes is repressed by H-NS, and mutations in hns result in various phenotypes, but the role of H-NS in the drug resistance of *Escherichia coli* has not been known. Here we present data showing that H-NS contributes to multidrug resistance by regulating the expression of multidrug exporter genes. Deletion of the hns gene from the _acrAB mutant increased levels of resistance against antibiotics, antiseptics, dyes, and detergents. Decreased accumulation of ethidium bromide and rhodamine 6G in the hns mutant compared to that in the parental strain was observed, suggesting the increased expression of some drug exporter(s) in this mutant. The increased drug resistance and decreased drug accumulation caused by the hns deletion were completely suppressed by deletion of the multifunctional outer membrane channel gene tolC. At least eight drug exporter systems require TolC for their functions. Among these, increased expression of acrEF, mdtEF, and emrKY was observed in the hns strain by quantitative real-time reverse transcription-PCR analysis. The hns-mediated multidrug resistance pattern is quite similar to that caused by overproduction of the AcrEF exporter. Deletion of the acrEF gene greatly suppressed the level of hns-mediated multidrug resistance. However, this strain still retained resistance to some compounds. The remainder of the multidrug resistance pattern was similar to that conferred by overproduction of the MdtEF exporter. Double deletion of the mdtEF and acrEF genes completely suppressed hns-mediated multidrug resistance, indicating that hns-mediated multidrug resistance is due to derepression of the acrEF and mdtEF drug exporter genes.

Olusegun O. Soge *et al* 2006 determined antibiotic resistance genes associated with 17 Nigerian CTX-M-positive*Klebsiella pneumoniae* plasmids from patients with community-acquired urinary tract infections Methods: The size and restriction patterns of the plasmids were determined, and antibiotic resistancegenes were identified using DNA–DNA hybridization, PCR assays, hybridization of PCR products withinternal probes, and sequencing.

Senka stated that 2007 since the discovery and subsequent widespread use of antibiotics, a variety of bacterialspecies of human and animal origin have developed numerousmechanisms that render bacteria resistant to some, and in certain cases to nearly all antibiotics. There are many important pathogens that are resistant to multiple antibiotic classes, and infections causedby multidrug resistant (MDR) organisms are limiting treatment options and compromising effective therapy. So the emergence of antibiotic-resistant pathogens in bacterial populationsis a relevant field of study in molecular and evolutionary biology, and in medical practice. There are two main aspects to the biology of antimicrobial resistance. One is concerned with the development, acquisition and spread of the resistance gene itself. The other is the specific biochemical mechanism conveyed by this resistance gene. In this review we present some recent data on molecular mechanisms of antibiotic resistance

Laura Paixao *etal*, 2009 stated that the Efflux pump activity has been associated with multidrug resistance phenotypes in bacteria, compromising the effectiveness of antimicrobial therapy. The development of methods for the early detection and quantification of drug transport across the bacterial cell wall is a tool essential to understand and overcome this type of drug resistance mechanism. This approach was developed to study the transport of the efflux pump substrate ethidium bromide (EtBr) across the cell envelope of *Escherichia coli* K-12 and derivatives, differing in the expression of their efflux systems.

Desj Simeoni 2007 stated that the *Staphylococci* harbouring antibiotic resistance (AR) genes may represent a hazard for human health and, as other resistant foodrelatedbacteria, they contribute to the spread of AR. In their study, they isolated resistant staphylococci from an entire swine production chain and investigated the occurrence of 11 genes [aac(60)Ie-aph(200)Ia, blaZ, mecA, andtet(K)] encoding resistance to some antibiotics largely used in clinical practice. The 66 resistant *staphylococcal* isolates were identified as *Staphylococcus epidermidis* (27 isolates), *Staphylococcus aureus* (, *Staphylococcus xylosus Staphylococcus* simulans *Staphylococcus pasteuri , Staphylococcus carnosus, Staphylococcus lentus* isolates *and Staphylococcus sciuri* . Specific-PCR detection of AR genes showed the prevalence of the tet(K) gene in most of the isolates (89.4%), followed by tet(M) and

ermC (about 75%); mecAwas detected in more than half of *S. aureus and S. epidermidis* isolates. The genes vanA and vanB were not retrieved. It was found that a high proportion of coagulase-positive and -negative isolates are multidrug-resistant and some of them carry up to six AR genes. Their findings show that the swine production chain is a source of antibiotic-resistant staphylococci suggesting the importance of resistances urveillance in the food production environment.

Anja S Hummel 2007 studied the antibiotic resistances of 45 lactic acid bacteria strains belonging to the genera Lactobacillus, Streptococcus, Lactococcus, Pediococcus, and Leuconostoc were investigated. Their objective was to determine antibiotic resistances and to verify these at the genetic level, as is currently suggested by the European “qualified presumption of safety” safety evaluation system for industrial starter strains. In addition, they sought to pinpoint possible problems in resistance determinations. Primers were used to PCR amplify genes involved in_lactam antibiotic, chloramphenicol, tetracycline, and erythromycin resistance. The presence of ribosomal protection protein genes and the ermB gene was also determined by using a gene probe. Generally, the incidences of erythromycin, chloramphenicol, tetracycline, or _lactam resistances in the study were low (<7%). In contrast, aminoglycoside (gentamicin and streptomycin) and ciprofloxacin resistances were higher than 70%, indicating that these may constitute intrinsic resistances. The genetic basis for ciprofloxacin resistance could not be verified, since no mutations typical of quinolone resistances were detected in the quinolone determining regions of the par C and gyrA genes. Some starter strains showed low-level ampicillin, penicillin, chloramphenicol, and tetracycline resistances, but no known resistance genes could be detected. Although some strains possessed the cat gene, none of these were phenotypically resistant to chloramphenicol. Using reverse transcription-PCR, these cat genes were shown to be silent under both inducing and non inducing conditions. Only *Lactobacillus salivarius* BFE 7441 possessed an ermB gene, which was encoded on the chromosome and which could not be transferred in filter-mating experiments. Their study clearly demonstrates problems encountered with resistance testing, in that the break point values are often in adequately identified, resistance genes may be present but silent, and the genetic basis and associated resistance mechanisms towardsome antibiotics are still unknown.

Neal C. stewart 1996 stated the somatic embryos of Jack, a Glycine max (1.) Merrill cultivar, were transformed using microprojectile bombardment with a synthetic *Bacillus thuringiensis* insecticidal crystal protein gene (BtcrylAc) driven by the 35s promoter and linked to the HPH gene. Approximately 10 g of tissue was bombarded, and three transgeniclines were selected on hygromycin-containing media and convertedinto plants. The recovered lines contained the HPHgene, but the Btgene was lost in one line. The plasmid was rearranged in the secondline, and the third line had two copies, one of which was rearranged.The CrylAc protein accumulated up to 46 ng mg-' extractableprotein. In detached-leaf bioassays, plants with an intact copy of the Bt gene, and to a lesser extent those with the rearranged copy, were protected from damage from corn ear worm *(Helicoverpazea)*, soybean looper *(Pseudoplusia includens)*, tobacco budworm *(Heliothis virescens)*, and velvetbean caterpillar *(Anticarsiagemmatalis)*. Corn earworm produced less than 3% defoliation ontransgenic plants, compared with 20% on the lepidopteran-resistant breeding line CatlR 81-296, and more than 40% on susceptible cultivars. Unlike previous reports of soybean transformation using this technique, all plants were fertile.

Notani. n. k. *et al* 1984 studied and reported that certain species of bacteria can become competent to take up high molecular weight DNA from the surrounding medium. DNA homologous to resident chromosomal DNA istransported, processed and recombined with the resident DNA. There are some variations insteps leading to transformation between Gram-positive bacteria like biplococcus pneumonia and Gram-negative bacteria represented by *Haemophilus influenzae* but the integration is bysingle-strand displacement in both cases. Plasmid (RSF0885)transformation is low in *Haemophilus influenzae* but this is increased significantly if (homologous) chromosomal DNAis spliced to plasmid DNA. In *Haemophilus influenzae*, rec1 function is required for peak transformation with chimeric plasmids. Chimeric plasmid fixed presumably extrachromosomally undergoes frequent recombination between homologous segments contained in resident chromosome and the plasmid.

Niimi *K. et al* 2005 the Micafungin and caspofungin susceptibilities of Candida albicans laboratory and clinical isolates and of *Saccharomyces cerevisiae* strains stably hyperexpressing fungal ATP-binding cassette (ABC) or major facilitator superfamily (MFS) transporters involved in azole resistance were determined using

three separate methods.by (Niimi *K).* yeast strains hyperexpressing individual alleles of ABC transporters or an MFS transporter from C. albicans gave the expected resistance profiles for the azoles fluconazole,itraconazole, and voriconazole. The strain shyperexpressing CDR2 showed slightly decreased susceptibility to caspofungin in agar plate drug resistance assays, as previously reported, but increased susceptibility to micafungin compared with either the strains hyperexpressing CDR1 or the null parent deleted of seven ABC transporters. The strains hyperexpressing CDR1 showed slightly decreased susceptibility to micafungin in these assays. *A C. albicans* clinical isolate overexpressing both Cdr1p and Cdr2p relative to its azole-sensitive isogenic progenitor acquired resistance toazole drugs and showed reduced susceptibility to caspofungin and slightly increased susceptibility to micafunginin agar plate drug resistance assays. None of the strains showed significant resistance to micafungin orcaspofungin in liquid microdilution susceptibility assays. The antifungal activities of micafungin and caspofunginwere similar in agarose diffusion assays, although the shape and size of the caspofungin inhibitory zoneswere affected by medium composition. The assessment of micafungin and caspofungin potency is therefore assay dependent; the differences seen with agar plate drug resistance assays occur over narrow ranges of echinocandin concentrations and are not of clinical significance.

Aure lie A. Huet 2008 stated the Biocides and dyes are commonly employed in hospital and laboratory settings. Many of these agents are substrates for multiple-drug resistance (MDR)-conferring efflux pumps of both Gram-positive and Gram-negative organisms. Several such pumps have been identified in *Staphylococcus aureus*, and mutants over expressing the NorA and MepA MDR pumps following exposure to fluoroquinolones have been identified. The effect of exposure to low concentrations of biocides and dyes on the expression of specific pump genes has not been evaluated. Using quantitative reverse-transcription PCR we found that exposure of clinical isolates to low concentrations of a variety of biocides and dyes in a single step, or to gradually increasing concentrations over several days, resulted in the appearance of mutants over expressing mepA, mdeA, norA and norC, with mepA over expression predominating. Over expression was frequently associated with promoter-region or regulatory protein mutations. Mutants having significant increases in MICs of common pump substrates but no changes in expression of studied pump genes were

also observed; in these cases changes in expression of as-yet-unidentified MDR pump genes may have occurred. Strains of *S. aureus* that exist in relatively protected environments and are repeatedly exposed to sublethal concentrations of biocides can develop efflux-related resistance to those agents, and acquisition of such strains poses a threat to patients treated with antimicrobial agents that are also substrates for those pumps, such as ciprofloxacin and moxifloxacin.

Mahipal Singh *et al* 2010 optimized various parameters of standard CaCl2/heat shock method on transformation of *Escherichia coli* strain DH5α -T1R with plasmid pUC19. Of the four different heat shock temperatures (32°C, 37°C, 42°C and 47°C) studied, 42°C treatment exhibited maximum efficiency of transformation as revealed by ampicillin-resistant colonies appearing on LB Agar ampicillin plates. Of the five different heat shock exposure times, a pulse of 30 second duration combined with 42°C heat shock temperature exhibited maximum efficiency. It was observed that although transformation of $CaCl_2$ treated cells occurs even before heat shock treatment, the efficiency was ~15 fold higher after heat shock. When the cells were further incubated on ice (after heatshock) for 10 min, the transformation efficiency increased by 24 fold compared to no heat shock and 1.6 fold compared to heat shock treatment. There was a marginal decrease in transformation when cells were incubated at room temperature instead of ice after heat shock. These results suggest that a heat shock pulse of 30 sec at 42°C followed by a 10 min ice incubation step are ideal parameters to obtain maximum transformation efficiency in DH5α -T1R strain. Results also suggest that post heat shock cold incubation step is also an important factor and enhances transformation of *E. coli* significantly

Ameena S. M. Jum studied a five hundred stool samples were collected from patients with diarrhea (infants and childrenunder ten years of age) admitted to the Pediatric and Maternity Hospital in Erbil City from March 2007 to September 2007. The samples were cultured on different culture media and according to the colony morphology, biochemical reactions and by the use of API 20E system, 35 (7%) were diagnosed as *E.coli I,* 8 (1.6%) *E.coli II*, 17 (3.4%) *E.coli I*II, 22 (4.4%) *E.coli IV,* 8 (1.6%) *Shigella dysenteriae*, 16 (3.2%) *Salmonella arizonae,* 12(2.4%) *Salmonella typhi* and 6 (1.2%) *Vibrio cholerae.* In addition, cases of *Entamoeba histolytica* 175 (35%),*Giardia lamblia* 102 (20.4%) and *Hymenolepis nana* 2 (2.4%) were identified.

No infectious agents were found in 75 (15%) of the samples. 22 (4.4%) of the samples had mixed infections. The sensitivity of *E.coli* O157:H7isolate to different antibiotics was performed. There was a variation in the resistance ranging from 8.5-90%.the determination of the site of genes responsible for the antibiotic resistance in *E.coli* O157:H7 was performed using the genetic transformation method for *E.coli DH5α* laboratory strain with the DNA that is absent from the highly resistant strains, *E.coli* O157:H7 4 and E. coli O157:H7 6. The transformation process succeeded when using the plasmid DNA for strain 4 and failed when using strain 6. It was evident that the genes responsible for resistance to the following antibiotics were located on the plasmid DNA: amoxicillin, amoxiclav, ampicillin, cephalexine, cefixime, cefotaxime, doxycyclin, gentamycin, nalidixic acid, nitrofurantoin, rifampicin, streptomycin and tetracycline. Whereas the genes responsible for the following antibiotic resistance were located on the chromosome: amikacin, erythromycin, chloramphenicol, ciprofloxacin, tobramycin and trimethoprim.

Douglas Hanahan *et al* 1983 stated the factors that affect the probability of genetic transformation of *Escherichia coli* by plasmids have been evaluated. A set of conditions is described under which about one in every 400 plasmid molecules produces a transformed cell. These conditions include cell growth in medium containing elevated levels of Mg 2+. and incubation of the cells at 0~ in a solution of Mn 2+, ("a 2+, Rb + or K +, dimethyl sulfoxide, dithiothreitol, and hexamine cobalt (III). Transibrmation efficiency declines linearly with increasing plasmid size. Relaxed and supercoiled plasmids transformation with similar probabilities. Non-transforming DNAs compete consistent with mass. No significant variation is observed between competing DNAs of different source, complexity, length or form. Competition with both transforming and non transforming plasmids indicates that each cell is capable of taking up many DNA molecules, and that the establishment of a transformation event is neither helped nor hindered significantly by the presence of multiple plasmids.

Sharon D. Cosloy and Michio Oishi 1972 studied the an auxotrophic strain of E. coli K12 treatedwith $CaCl_2$ was transformed for several markers at a frequency of up to 6 per recipient cell by a DNA preparation isolated from a prototrophic strain. The transforming activity of the DNA preparation was eliminated by treatment with

DNase, heat, or sonication, whereas RNse or Pronase treatment had little effect. Two closely linked genetic markers (leu and ara) showed a high degree of co transformation linkage when high molecular weight DNA was used, but the linkage was almost completely eliminated when sheared, smaller molecular weight DNA was used. There is genetic evidence that the transformation is a result of the replacement of the preexisting genetic marker on the chromosome by that of the donor DNA.

Glenn W. Kaatz 2000 *et al* created the *Staphylococcus aureus norA* disruption mutant by allelic replacement. Exposure of this mutan to norfloxacin produced SA K1748, a derivative with raised fluoroquinolone MICs, found to be the result of a*grlA* mutation, and raised organic cation MICs. Ethidium and enoxacin uptake was identical in SA K1748 and its parent, but pre-exposure of SA K1748 to organic cations caused a reduction in ethidium uptake as a result of increased efflux. Altered ethidium uptake and efflux, as well as increased MICs of other organic cations, suggest that SA K1748 possesses a non-NorA multidrug efflux transporter that is inducible by its substrates. Factors that affect the probability of genetic transformation

Hiroshi Nikaido 2009 studied the large amounts of antibiotics used for human therapy, as well as for farm animals and even for fishin aquaculture, resulted in the selection of pathogenic bacteria resistant to multiple drugs. Multidrugresistance in bacteria may be generated by one of two mechanisms. First, these bacteria may accumulate multiple genes, each coding for resistance to a single drug, within a single cell. This accumulation occurs typically on resistance (R) plasmids. Second, multidrug resistance may also occur by the increased expression of genes that code for multidrug efflux pumps, extruding a widerange of drugs.

Magdalena Chroma 2010 reviewed and reported the emergence of antibiotic resistance. currently, bacterial resistance to antibiotics poses a major problem in both hospital and community settings throughout the world.

David Dubnau 1999 stated that the natural competence is widespread among bacterial species. The mechanism of DNA uptake in both gram-positive and gram-negative bacteria was also reviewed. By their Uptake of DNA across the inner membrane is probably similar in gram positive and gram-negative bacteria, and at

least some of the required proteins are orthologs. The initial transformation steps differ, as expected, from the presence of an outer membrane only in the gram-negative organisms. The similarity of certain essential competence proteins to those required for the assembly of type-4 pili and fortype-2 protein secretion is discussed. Finally several hypotheses for the biological role of transformation are presented and evaluated by their.

Simeon Oloni Kotchoni 2003 developed a rapid protocol for plasmid DNA extraction based on the alkaline lysis method of plasmid preparation (extraction at pH 8.0). Instead old method which is a five coming using this new method, a good plasmid preparation can be made in approximately one hour. The plasmids are suitable for any subsequent molecular applications in the laboratory. By applying the recommendations to avoid contaminations and to maximize the plasmid yield and quality during extraction, their protocol could be a valuable reference especially when analyzing a large number of samples**.**

Thomas P Quinn and Katrina T Trevor 1997 Isolation of low-molecular-weight DNA from eukaryotic cells entails the separation of a very small quantity of plasmid DNA from a much larger quantity of genomic DNA. Traditionally, the Hirt extraction method (3) has been used for this purpose. This method, however, is labor-intensive, involving extensive precipitations andphenol/chloroform extractions. In their report, three alternative methods are compared for the isolation of episomalc DNA expression vectors containing the human interleukin-2 (*IL-2*) or interferon-g (*IFN-g*) gene from T lymphocytesthat were stably transfected with the corresponding constructs: *(i)*a boilingmethod (4), *(ii)*alkaline lysis basedon the method of Birnboim and Doly(1) in combination with a phenol/chloroform extraction and ethanol precipitationand *(iii)*the QIAprepÒ procedure (Qiagen, Chatsworth, CA, USA), which combines alkaline lysis of the samples with an adsorption of plasmid DNAonto a silica matrix. All procedures were originally designed for plasmid preparations from *Escherichia coli*

Amin *et al* 2009 reported the antibiotic resistance pattern and extended spectrum β-lactamase production is cultures of forty *Klebsiella pneumoniae* isolates isolated in a Tertiary Care Hospital in Islamabad Pakistan towards novel

cephalosporin (first, second and third generation), floroquinolones, carbapenems, amoxicillin/clavulanic acid and lincomycin by disc sensitivity, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Cephalosporin was reported as highly resistant (cephradine 100%, cephalexin 75%, cefeclor 87.5%, ceftriaxone 85%, cefotaxime 82.5%), lincomycin (100%), followed by quinolones (ciprofloxacin 55%, ofloxacin 47.5%, nalidixic acid 42.5%, norfloxacin 35%,moxifloxacin 25%, gatifloxacin 15%), amoxicillin/clavulanic acid 12.5%, and carbapenams (imipenem, meropenem) with the least resistance at 7.5%. About 47.5% strains were found to be ESBLs positive among which 15.8% of the strains were producing carbapenamase, 26.5% with inducible cephalosporinase of bush functional gene 2e. No inhibitor resistant TEM-β lactamases (IRT) positive strains were found. High antibiotic resistance rate against commonly use dantibiotics is a disadvantage for health care system in countries like Pakistan as it can greatly effect patient management. Therefore they recommend physicians must change prescription priorities towards alternative antibiotics to reduce the burden of antibiotics resistance.

Lateef.A *et al*2003 studied the resistance pattern and mechanisms of bacterial isolates obtained from clinical origin, soil, industrial effluent, orange juice products and drinking water were studied using commonly used antibiotics. The microbial load of the water samples, industrial effluent and orange juice products were 1.0×10–2.25×10, 2.15×10, and 3.5×104.15×10 cfu mL, respectively. The faecal coliform test revealed that only two out of twenty orange juice products had MPN of 2 and20, the MPN of water ranged from 1800, while the effluent had MPN of ≥1800. The bacterial isolates that were isolated include *E. coli, S. aureus, P. vulgaris, S. marcescens, S. pyogenes, B.cereus, B. subtilis, Micrococcus* sp., *Klebsiella* sp., *P. aeruginosa*, and *Enterobacter* sp. Also, clinical and soil isolates of *P. aeruginosa* were used in the study. Among the eight antibiotics tested for resistance on five strains of each bacterium, seven different resistance patterns were observed among the bacterial isolates obtained from water, effluent and orange juice products. Among the clinical and soil isolates of *P. aeruginosa*, four multiple-drug resistance patterns were obtained. Thirty strains of *E. coli* and *S. aureus* were tested for *β*-lactamase production and fourteen strains, seven each of *E. coli* and *S. aureus* that had high Minimum Inhibitory Concentration values (MIC) for both Amoxycillin and Cloxacillin were positive.

Asma Bashir *et al (2007)* stated that although most a bacterial infections of the skin bear out to be minor in nature, a few such dermatologic entities are major, to the spot of yet being fatal. The mortality rate is usually up to 30% to 50% and depends upon the type of infection, original disease, and resistant type. In this study hundred and five bacterial strains were isolated from skin wounds, burns and acne patients from hospitals at different locations in the cosmopolitan city of Karachi. These bacterial strains were identified by conventional methods. Seventy two percent (72%) of total isolated organisms were found to be *Staphylococcus aureus* while the remaining thirty three percent (33%) were *Staphylococcus epidermidis.* The antibiotic resistance of identified organisms was carried out by disc-diffusion method with commercially available disc of five antibiotics having different mode of actions such as cell wall synthesis inhibitors, membrane permeability alternatives and DNA synthesis inhibitors. *Staphylococcus aureus*show more resistant to these antibiotics as compared to *Staphylococcus epidermidis.* The most effective antibiotic for *Staphylococcus aureus* is vancomycin showing 80.5% efficacy, then methicillin with 68.0% efficacy, erythromycin with 55.6% efficacy, novobiocin with 54.1% efficacy and then bacitracin with 25.0% efficacy. The most effective antibiotic for *Staphylococcus epidermidis* is methicillin showing 84.8% efficacy,then vancomycin with 81.2% efficacy, novobiocin with 63.6% efficacy, erythromycin with 42.4% efficacy and then bacitracin with 27.8% efficacy.

Melanie P Dautle *et al* 2004 performed in vitro analysis of 7 antibiotics against100 clinical bacterial isolates enriched from pediatric gastrostomy tubes. Various gram positive and negative organisms were purified from percutaneous pediatric feeding tubes and assayed for antibiotic susceptibility and resistance. A total of10 gram-negative isolates, Predominantl *Escherichia coli,* and 90 gram-positive organisms, mostly belonging to the *Staphylococcus* and *Enterococcus* genuses were examined for antimicrobial resistance and sensitivity. Seven antibiotics, which included ampicillin, cefazolin, ceftriaxone, ciprofloxacin, gentamicin, oxacillin, and vancomycin, were tested based on their possible usein pediatric patients requiring feeding tubes for nutritional support. Minim inhibitory concentrations were determined for all isolates and their relative resistance profiles were generated. The *Staphylococcus* genus possessed the highest diversity for antibiotic resistance while

organisms comprising the *Enterococcus* genus exhibited marginal levels of resistance to the antibiotics tested in this study. Approximatel43% of the isolates tested displayed multiple drug resistance, with the predominant species belonging to the *Staphylococcus* genus. This investigation reports the effectiveness of 7 commonly used antibiotics on various microbial species that are capable of initiating and maintaining bacterial biofilms on surgically implanted feeding tubes.

Mustafa Oskay et al 2009 reported the plants used for traditional medicine contain a wide range of substances which can be used to treat various infectious diseases. Hence, antibacterial activities of ethanolic extract of 19 plant species were studied against multi-drug resistant clinical isolates using agar well diffusion method. Extracts of Liquidambar oriental is, Vitisvinifera, Punica granatum, Cornus sanguinea, Euphorbia peplus, *Ecballium elaterium*, Inula viscose and Liquidambar oriental is showed broad-spectrum antibacterial activity with inhibition zones ranging from 8 to 26 mm. The most resistant organisms were *Escherichia coli* (*E.coli*) (Ampicillin-, amoxycillin- and sulfamethoxazole-resistant), *Stenotrophomonas* Maltophilia (*S.maltophilia*) (Amoxycillin- and nalidixic acid-resistant) and *Klebsiella pneumoniae* (*K.pneumoniae*) (Ampicillin-, amoxycillin- and aztreonam-resistant), and the most susceptiblespecies were *Staphylococcus aureus* (*S. aureus*) (Penicillin G- and oxacillin-resistant), *Streptococcus pyogenes* (*S. pyogenes*) (Penicillin G-, erythromycin- and clindamycin-resistant) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Sulfamethoxazole- and novobiocin-resistant), respectively. Minimum Inhibitory Concentrations (MIC) of crude extracts were determined forthe seven highly active plants showing activity against methicillin resistant *S. aureus* (MRSA),E. coli, *P. aeruginosa*, *S. pneumoniae* and the reference bacteria (E. coli ATCC 11229 and Kocuria rhizophila ATCC 9341 NA). MICs of active extracts ranged from 8 to 14.2 mg/mL against one or other test bacteria.

Adenike A. O. Ogunshe 2006 studied the massive problems facing the primary health care system in Nigeria, the consumer preference for indigenous herbal medications is on the increase. Since it is possibility that herbal medications may harbour pathogenic bacteria of clinical significance, the study determined the phenotypic antibiotic pattern of associated bacterial isolates using modified agar disc-diffusion and agar well-diffusion methods. All isolated bacterial species exhibited

mono and/or multiple *in vitro* phenotypic antibiotic resistance to the test antibiotic (discs). High resistance patterns were also observed towards paediatric antibiotic suspensions. This study confirmed that most indigenous orally consumed herbal medications in Nigeria harbour bacterial flora that exhibit multiple resistance to routinely used antibiotics.

Srisurang Tantimavanich 2002 stated the streptococci that were dependent for their growth upon staphylococci were isolated from a patient with sub-acute bacterial endocarditis and subsequently identified as nutritionally variant *streptococc*i (NVS). Failure of the isolate to grow on agar media supplemented with pyridoxal hydrochloride or L-cysteine, the known supporting growth factors for NVS, made conventional antimicrobial disc diffusion assay impossible. We modified the assay by co-inoculating *Staphylococcus aureus* resistant to the drugs being tested as a helper to support the growth of the NVS. Streaking *S. aureus* closely to the antibiotic discs that were placed above NVS resulted in the growth of satellite colonies of NVS that orbited the *S. aureus* and that produced a pattern of interrupted zones of growth inhibition. Using an alternative method – adding staphylococcal secreting factor(s) to a 10% staphylococcal cell-free culture supernatant and adding this to an antibiotic susceptibility testing medium, – we found that the NVS formed colonies that formed clear zones of growth inhibition around the disc. When the sizes of the growth inhibition zones produced by both these methods were compared with those recommened by the NCCLS, the NVS were found to be susceptible to penicillin, vancomycin, erythromycin, chloramphenicol, cefoperazone, cefamandole and ofloxacin and resistant to co-trimoxazole, gentamicin and tetracycline. Based on the findings, vancomycin was selected for treatment and the patient was cured of endocarditis. The correlation between the *in vitro* drug susceptibility testing and the *in vivo* clinical response indicated that the modified antibiotic susceptibility test is an appropriate method for establishing antibiotic regimens significantly by the presence of multiple plasmid

Gulcan ozbakir *et al 2010* studied the *E. coli* strains, isolated from different clinical materials, were analysed according to their antibiotic resistance patterns and plasmid DNA profiles. When the incidence of *E. coli* infections are considered, more *E. coli* infections were observed in females than in males. Of the all *E. coli* strains isolated from clinical materials, 27% were resistant to only one antibiotic, 25% were

resistant to 6 antibiotics and resistance to all 8 antibiotics was established as 6%. In addition to these, resistance rate was highest for ampicillin and ciprofloxacin, whereas lowest resistance was displayed against Piperacillin/Tazobactam. According to the antibiotic resistance patterns of *E. coli* strains, 10 different antibiotypes were generated and the one, which was observed in highest frequency includes ampicillin–resistant property in itself (antibiotype 1). When the plasmid profiles of *E. coli* strains are examined, the profile which includes single band was observed as the most frequent one. It was also established that most of the *E. coli* strains, which were classified in that plasmid profile, acquire the properties of antibiotype 1. Besides it was also observed that; some of the *E. coli* strains, which do not contain any plasmid in their cells showed resistance to various antibiotics.

Hleba L, Kacaniova.M, *et al 2011*,statet the antibiotic resistance was investigated in 67 samples of *Enterobacteriaceae* genera isolates from milk, cheese and other dairy products in this work. The samples were obtained from conventional farms in Slovakia. Four samples of isolates were positive for *Salmonella* spp., *Salmonella enterica* ser. typhimurium and enteritidis. A great proportion of resistant strain from *Enterobacteriaceae* genera was found. There was detected a high resistance in milk samples to ampicillin (57.14%), to streptomycin and tetracycline (14.28%), to chloramphenicol (9.52%). Bacteria from the cheese samples were resistant to ampicillin (84.0%), to tetracycline (24.0%). In the samples of other dairy products was found resistance to ampicillin (66.66%), to tetracycline (52.38%) and to streptomycin (14.28%). Among all samples observed, it was found resistance to ampicillin (69.26%), tetracycline (30.22%), streptomycin (9.52%) and to chloramphenicol (3.17%). Resistance to other antibiotics was not detected. From all observed samples we detected *Salmonella* spp. in 5.97 %, *Salmonella* spp. was found in dairy samples in 19.04 %. Antibiotic resistance was investigated in 13 colonies of *Salmonella* spp. as well. The highest resistance was to tetracycline (30.76 %), ampicillin and tigecycline (23.07 %), to piperacillin (15.38 %) and chloramphenicol (7.69 %). Resistance to the other monitored antibiotics was not detected. High number of antibiotic

Keith Poole 2001 studied Pseudomonas aeruginosa is an opportunistic human pathogen characterized by an innate resistance to multiple antimicrobial agents. A

major contribution to this intrinsic multidrug resistance is provided by a number of broadly-specific multidrug efflux systems, including MexAB-OprM and MexXY-OprM. In addition, these and two additional tripartite efflux systems, MexCD-OprJ and MexEF-OprN, promote acquired multidrug resistance as a result of mutational hyperexpression of the efflux genes. In addition to antibiotics, these pumps promote export of numerous dyes, detergents, inhibitors, disinfectants, organic solvents and homoserine lactones involved in quorum sensing. The efflux pump proteins are highly homologous and consist of a cytoplasmic membraneassociated drug-proton antiporter of the Resistance - Nodulation-Division (RND) family, an outer membrane channel-forming protein [sometimes called outer membrane factor (OMF)] and a periplasmic membrane fusion protein (MFP). Homologues of these systems have been described in Stenotrophomonas maltophilia, Burkholderia cepacia, Burkholderia pseudomallei and the non-pathogen Pseudomonas putida, where they play a role in export of and resistance to multiple antimicrobial agents and/or organic solvents. Although the natural function of these multidrug efflux systems is largely unknown, their contribution to antibiotic resistance and their conservation in a number of important human pathogens makes them logical targets for therapeutic intervention.

Didier Hocquet 2007 stated investigated the resistance mechanisms to lactams, aminoglycosides, and fluoroquinolones of 120 bacteremic strains of *Pseudomonas aeruginosa*. Pulsed-field gel electrophoresis genotyping showed that 97 of these strains were represented by a single isolate, 10 by 2 and 1 by 3 clonally related isolates, respectively. Seventy-five percent (90 out of 120) of the bacteremic *P. aeruginosa* strains displayed a significant resistance to one or more of the tested antimicrobials (up to 11 for 1 strain). These strains were found to harbor a great diversity of resistance mechanisms (up to 7 in 1 strain), leading to various levels of drug resistance. Interestingly, 11 and 36% of the isolates appeared to overproduce the MexAB-OprM and MexXY-OprM efflux systems, respectively. Altogether, our results show that *P. aeruginosa* may accumulate intrinsic (overproduction of cephalosporinase AmpC, increased drug efflux, fluoroquinolone target mutations, and deficient production of porin OprD) and exogenous (production of secondary lactamases and aminoglycoside- modifying enzymes) resistance mechanisms without losing its ability to generate severe bloodstream infections. Consequently, clinicians should be aware that multidrug-resistant *P. aeruginosa* may remain fully pathogenic

Tanya Strateva And Daniel Yordanov 2009, Pseudomonas aeruginosa nosocomial Nosocomial infections caused by this organism are often hard to treat because of both the intrinsic resistance of the species (it has constitutive expression of AmpC b-lactamase and efflux pumps, combined with a low permeability of the outer membrane), and its remarkable ability to acquire further resistance mechanisms to multiple groups of antimicrobial agents, including b-lactams, aminoglycosides and fluoroquinolones. P. eruginosa represents a phenomenon of bacterial resistance, since practically all known mechanisms of antimicrobial resistance can be seen in it: derepression of chromosomal AmpC cephalosporinase; production of plasmid or integronmediated b-lactamases from different molecular classes (carbenicillinases and extendedspectrum b-lactamases belonging to class A, class D oxacillinases and class B carbapenemhydrolysing enzymes); diminished outer membrane permeability (loss of OprD proteins); overexpression of active efflux systems with wide substrate profiles; synthesis of aminoglycosidemodifying enzymes (phosphoryltransferases, acetyltransferases and adenylyltransferases); and structural alterations of topoisomerases II and IV determining quinolone resistance. Worryingly, these mechanisms are often present simultaneously, thereby conferring multiresistant phenotypes. This review describes the known resistance mechanisms in P. aeruginosa to the most frequently administrated antipseudomonal antibiotics: b-lactams, aminoglycosides and fluoroquinolones

3. AIM OF WORK

- To understand the microbial resistance towards the selected antibiotics.
- To determine the ability of microbes to transfer resistance characters.
- To isolate plasmid and determine the molecular weight.
- To understand the mechanism (efflux pump) of the clinical isolates to resist antibiotic.

4. PLAN OF WORK

Collection of clinical bacterial isolates and antibiotics

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Determination of MIC and Susceptibility analysis

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Isolation of plasmid DNA from resistant bacteria

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Agarose Gel Electrophoresis

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Transformation assay

↓

Efflux pump analysis

5. Material and methods

5.1. Table 1 List of Chemicals & Media

S.No	Chemical	Company
1	Ampicillin disc	Himedia
2	Chloromphenicol disc	Hi media
3	Roxithromycin disc	Himedia
4	Ceftriaxone disc	Himedia
5	Nutrient broth	Himedia
6	Nutrient agar	Himedia
7	Muller hinton agar	Himedia
8	Resazurin dye	Himedia
9	Ethidium bromide	SRL
10	Calcium chloride	Ottokemi
11	Glucose	Nice Chem
12	Isopropanol	Finar

5.2. Table 2 Strains

S.No	Culture	Obtained from laboratory
1	*Pseudomonas aeruginosa*	Vijay laboratory
2	*Staphloccocus aeurus*	Vijay laboratory

5.3. Table 3 List of Instrument Used

S.No	Instruments	Manufacture
1	Incubator rotary shaker	Scigenics Orbitek
2	Incubator	ILTS
3	Centrifuge	Remi R24
4	Autoclave	Equitron
5	Pipette	Qualigens
6	Beaker	Qualigens
7	Hot air oven	Ausco
8	Centrifuge tubes	Tarsons
9	Measuring cylinder	Qualigens
10.	pH meter	Shimadzu
11.	Erlenmeyer flask	Qualigens
12.	Digital balance	Shimadzu AY220
13.	Colorimeter	Aimil ltd
14.	Water bath	ITC
15.	Vortex	Spinix
16.	Conical flask	Qulaigens
17.	Refrigerator	LG
18.	Electrophoresis tank	Aplex

5. METHODS

5.1 Collection of clinical isolated bacteria and antibiotic

The clinical isolated bacteria *Pseudomonas aeruginosa* and *Staphyloccocus aureus* obtained from Vijay clinical laboratory Madurai and antibiotic discs used ampicillin, chloromphenicol, ceftriaxone and roxithromycin were obtained from media, Mumbai.

5.2. Susceptibility

Susceptibility tests were performed by Kirby Bauer technique (Cappucino 2007) and the results were observed from Photo 1&2.

Procedure

1. Agar plates was placed right side up in an incubator heated to 37C for 10 to 20 minutes with covers adjust so that the plates were slightly opened
2. Covers of each plates was labeled with the name of the test organism to be inoculated.
3. Using sterile technique ,all agar plate were inoculated with their respective test organisms as follows:

A sterile cotton swab was dipped into a well –mixed saline test culture and excess inoculum was removed by pressing the saturated swab against the inner wall of the culture tube.

Using the swab, the entire agar surface was streaked horizontally, vertically, and around the outer edge of the plates to ensure a heavy growth over entire surface.

4. All culture plates were allowed to dry for about 5 minutes.
5. Using the forceps the antibiotic disc were placed over the agar surface and pressed gently to ensure that the disc adhere to the surface of the agar.
6. All plate culture were incubated in an inverted position for 24 hr at 37^{o}C
7. Table 4 showed the susceptibility of isolated microorganisms to various antibiotic.

5.3 .Minimum inhibitory concentration

The MIC was performed by resazurin microtitre assay (Satyajit D. Sarker 2007) and results were tabulated in Table 5 and Photo 3, 4, 5 & 6.

Preparation of resazurin solution

The resazurin solution was prepared by dissolving 62 mg in 10 mL of sterile distilled water. A vortex mixer was used to obtain a well-dissolved and homogenous solution.

Preparation of the plates

Plates were prepared under aseptic conditions. A sterile 96 well plate was labelled.

The antibiotic used concentration 1mg/ml and perform serial dilution 0.5 Serial dilutions were performed using a multichannel pipette. To all wells add 100 ul from the serially diluted antibiotics. To all wells 50 µL of nutrient broth was added and tips discarded after use. To each well 10 µL of resazurin indicator solution was added.

Finally, 10 µL of bacterial culture was added in and plates were plate wrapped loosely with cling film to ensure that bacteria did not become dehydrated.

Control plate contain antibiotic serially dilution + broth + aline +indicator (Satyajit D. Sarker 2007)

5.4 Plasmid DNA Preparation.

By alkaline lysis method.

1.5 ml of overnight culture centrifuge at 8000 rpm for 10 minutes, to the cell pellets were resuspended in 90 L GTE buffer (50mM glucose, 25 mM Tris-HCl, pH 8.0and 10 mM EDTA). After an incubation period of 5 min at room temperature, the cells were lysed by adding 90L lysis buffer (200 mM NaOH,1% sodium dodecyl sulfate [SDS] PH.9).Samples were mixed and placed on ice for 5 min. The solution was neutralized by adding 150 µL potassium acetate (KOAc) solution (5 M KOAc, pH 4.8 or 5.5). Cell debris and chromosomal DNA were subsequently removed from the solution by centrifugation (5 min at10000 rpm). Episomal DNA was further purified by iso-propanol, phenol / chloroform extraction and ethanol precipitation.

then dry the pellet and suspend in 20 L of TE buffer(Simeon Oloni Kotchoni *et al* 2003,, , Thomas P. Quinn *et al* 1997)

5.5. Agarose gel electrophoresis

Agarose gel 0.8% was prepared in 100 mL 0.5 X TE buffer and boiled, this was allowed to cool to 50ºC and 2 µL (1 mg/mL) of ethidium bromide was added. The gel was poured and comb was inserted to make the walls. When the gel was set, the comb was removed and the gel was placed in the gel box and immersed in 0.5 X TE buffer. Samples were prepared by taking 10 µL of the DNA, 2 µL of loading dye. Thegels were run for 90 min at 50 Volt, the gels were viewed and photographed (Photo 7) (Adel K K and Sabiha, S.S 2010)

5.6. Competent cell

5 ml of nutrient broth was inoculated with a single colony of *E.coli* DH5α (whose plasmid is manipulated genetically, plasmid less), incubated with shaking at 100 rpm for 24 hours at 37°C, then 1ml of bacterial culture was added to10 ml nutrient broth, incubated with shaking at 37°C, 100 rpm for 3-4 hours till OD become 0.3_o.5. The cells were harvested by centrifugation at 800 rpm, and then the pellet resuspended in 1.5 ml of cooled transformation calcium chloride keep in ice for 10 minutes and centrifuge at 500 rpm for 5 minute, , there suspended cell pellet in 1.5 ml of calcium chloride left on ice for 10 minutes, centrifuged for 5minutes at the same velocity, and resuspended in 0.2ml OD calcium chloride and glycerol and store. (Ameena SM Juma 2010)

5.7. Transformation process

2 µl of prepared plasmid DNA was added to the tube containing of 100µl competent cells. The mixture was placed on ice for 30 minutes, and then exposed to heat shock at 42°C for 2 minutes. 1ml of fresh nutrient broth was then added to transformation mixture and incubated at 37°C for 60 minutes to allow the expression of antibiotic resistant genes.

To the nutrient agar added the antibiotic use (chloromphenicol and ampicillin) and mixed well and then poured in plates to the plates streak or spread 0.1ml from the transformation mixture in nutrient agar contain appropriate antibiotic, and 0.1 ml competent cell and spread or streak on nutrient agar plates and the antibiotic used as control by using DH5 α . All plates were incubated at 37°C for 24 hours (Ameena S.

M. Juma 2010). The colonies of transformed culture were observed after incubation (Photo 8, 9, 10 & 11) and colony numbers were tabulated in Table 6.

5.8.Preliminary Efflux Pump Assay

Accretion of Ethdium bromide:

By the bacterial isolate in use. The presence of antibiotic use was done according to Bacterial cells were spotted on solid Nutrient agar medium(control) and Nutrient agar medium supplemented with 100 µg/ml of ethidium bromide. The plates were examined under UV accrual of ethidium bromide in bacterial cells after 24 hr incubation at 28^{o} C (Laura Paixao 2009, Lăzăroaie MM 2009, Edward A et al 2012)

5.9. Ethidium bromide Accumulation Assay:

To determine the efflux pumps activity, the accumulation assay of Ethidium bromide was performed with10 ml of early Log phase culture centrifuged and the supernatant was discarded. The pellets were washed twice with phosphate buffer saline (PBS) to remove the cell debris and other contaminants. Finally 10ml of PBS was added to the pellet and vortexed well. 20µl of Ethidium bromide was taken from stock solution (1mg/1ml), added to the 10ml of bacterial suspension and kept in rotrotary shaker at 150 rpm. At every 15 min interval, 1ml of culture was taken and centrifuged at 10,000 rpm for 5 min. Cell free supernatant was taken and OD value was measured at 600 nm (Edward A et al 2012, Kunihiko Nishino *et al* 2004). The accumulation plate were given in Photo 12, 13, 14 & 15 and results were tabulated in Table 7 and Chart 1.

5.10. Ethidium bromide Efflux Assay:

To confirm the efflux pumps activity, the efflux assay was performed using Ethidium bromide. 10 ml of early log phase culture was centrifuged and the supernatant was discarded. The pellets were washed twice and suspended in 10ml of PBS with increasing concentration of glucose (10, 50, 100 and 1000mM). 20µl of Ethidium bromide stock solution (1mg/1ml) was added to the culture and shaken at 150 rpm for 1h. At every 15 min regular interval, 1ml of culture was taken and centrifuged at 10,000 rpm for 5 min 1ml of supernatant was taken and OD values were observed at 600 nm (Kunihiko Nishino *et al* 2004, Edward.A, et al 2012).

The effect of Glucose concentration in efflux pump assay were tabulated in table.8, and expressed in Chart 2&3 for *Staphyloccocus aureus* and *Pseudomonas aeruginosa* respectively.

6. RESULTS AND DISCUSSIONS

Infections have been the major cause of disease throughout the history of human population. With the introduction of antibiotics, it was thought that this problem should disappear. However, bacteria have been able to evolve to become resistant to antibiotics (Senka D, and Jagoda *et al 2007*). It is clear that bacteria will continue to develop resistance to currently available antibacterial drugs by either new mutations or the exchange of genetic information, that is, putting old resistance genes into new hosts (Fred C.Tenover 2006). Also the overwhelming use of antibiotics has played a significant role in the outspread/emergence of antibiotic resistance bacteria. Antibiotics added to animal-feed and given to livestock that are used as human food contribute to additional resistance (Ashraf.R and Shah ,N.P 2011). In many healthcare facilities around the world, bacterial pathogens that express multiple resistance mechanisms are becoming the norm, complicating treatment and increasing both human morbidity and financial costs. Prudent use of antibacterial drugs using the appropriate drug at the appropriate dosage and for the appropriate duration is one important means of reducing the resistant organisms to emerge. New antibacterial agents with different mechanisms of action are also needed. It is difficult to outsmart organisms that have had several billion years to learn how to adapt to hostile environments, such as those containing antimicrobial agents.

In this experimental study the strains collected from clinical laboratory which resist to get controlled through antibiotics used were considered to determine their resistant pattern towards the respective multiple antibiotics. Initially the selected strains were screened for their susceptability towards the antibiotics listed herewith: Ampicillin, Chloramphenicol, Ceftriaxone and Roxithromycin using disc diffusion method. The evaluation of components as antimicrobial substance can be carried out by disc diffusion method described by Kirby Bauer. A standardized filter- paper disc-agar diffusion procedure is known as Kirby Bauer technique, which allows determining the drug susceptibility of microorganisms by means of rapid determination of the efficacy of drug by measuring the zone of inhibition diameter

that results from diffusion of the agent into the medium surrounding the disc. In this procedure, filter paper discs of uniform size are impregnated with specified concentrations of different component and then placed on the surface of an agar plate that has been seeded with the organism to be tested. The medium of choice is Muller Hinton Agar, with a pH of 7.2-7.4, whichates is aright gel where the different of drug facilitate carier following incubation, the plates are examined for the presence of growth inhibition, which is indicated by a clear zone surrounding each disc. The susceptibility of an organism to a drug is determined by the size of this zone, which itself is dependent on variables such as, the ability and rate of diffusion of the antibiotic into the medium and its interaction with the test organism, the number of organisms inoculated, the growth rate of the organism, the degree of susceptibility of the organism to the antibiotic. A measurement of the diameter of the zone of inhibition in millimeters is made, and its size is compared to that contained in a standardized chart NCCLS. Based on this comparison, the test organism is determined to be resistant, intermediate, or susceptible to the antibiotic.

Pseudomonas aeruginosa OCHM432 had antibiotic resistance of 62.5% to all the tested antibiotics while *Enterobacter aerogenes* OCHM11, OCHM7, *Klebsiella pneumoniae* OCHM26, OCHM263, *Escherichia coli* OCHM29 and *Shigella dysenteriae* OCHM39 had the lowest antibiotic resistance of 12.5%. (Adenike A *et al* 2006). It is very well understood from the study that *Pseudomonas aeruginosa* is developing resistance widely.

Staphylococcus aureus and *Staphylococcus epidermidis* were found to be sensitive against most of the antibiotics whereas *Staphylococcus aureus* show more resistance as compare to *Staphylococcus epidermidis (*Asma basher 2007*)*. These results showed the emergence of antibiotic resistance by microbes in diversified geographical accessions and particularly by *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

Table 4 clearly depicts that ampicillin is resisted by *P.aeuroginosa* but intermediate to chloramphenicol and chloramphenicol is resisted by *S.aureus* but intermediate to ampicillin. *P.aeuroginosa* and *S.aureus* were sensitive to both the ceftriaxone and roxithromycin. From these observations we decided to evaluate the

minimum inhibitory concentration of these antibiotics to the selected strains. This was done by rezasurin dye method.

Table 4: Susceptibility analysis			
S. No	**Antibiotics**	**Zone of Inhibition (mm)**	
		S. aureus	***P. aeuroginosa***
1	Ampicillin	14.1 ± 0.10	7.83 ± 0.08
2	Chloramphenicol	11.45 ± 0.29	14.97 ± 0.09
3	Ceftriaxone	23.97 ± 0.15	17.03 ± 0.14
4	Roxithromycin	22.87 ± 0.09	24.03 ± 0.20
n=3, ± SEM			

Photo 1

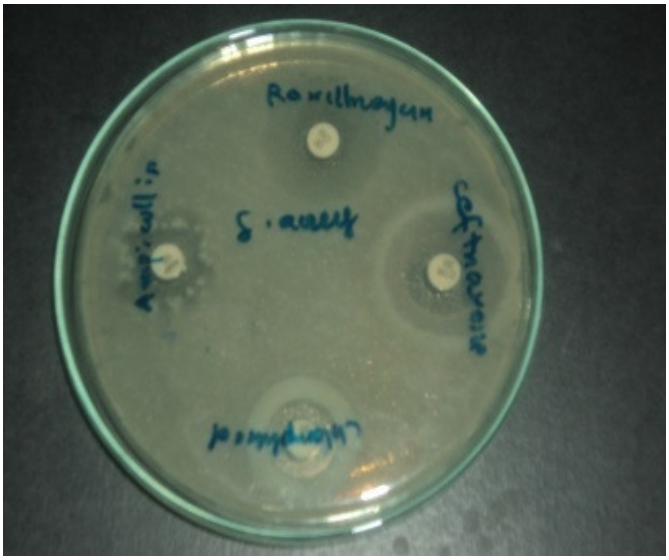


Photo 2

***S. aureus* sensitivity assay to various antibiotic**

***P. aeruginosa* Sensitivity assay to various antibiotic**

Resazurin is an oxidation–reduction indicator used for the evaluation of cell growth, particularly in various cytotoxicity assays. It is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. Resorufin is further reduced to hydroresorufin (uncoloured and nonfluorescent). A resazurin reduction test has also been used for decades to demonstrate bacterial and yeast contamination of milk (Sattyajit D,Sarker *et al* 2007), here in this study resazurin dye purchased from himedia is utilized where initial colour upon addition with culture showed blue. The colour change from blue to pink shows the growth of microbe in the well and the colour change from blue to pink indicate the minimum inhibitor concentration

Table 5: Minimum inhibitory concentration (µg/ml)

S.No	Antibiotic	*S. aureus*	*P. aeuroginosa*
1	Ampicillin	7.81	250
2	Chloramphenicol	0	7.81
3	Ceftriaxone	31.5	7.81
4	Roxithromycin	62.5	15.62

From the above table 8, we could observe that *P.aeuroginosa* is highly sensitive to ceftriaoxne (7.81µg/ml), chloramphenicol (7.81 µg/ml) and roxithromycin (16.62 µg/ml) while *S.aureus* is sensitive to ampicillin (7.81 µg/ml) alone. When we compare these results with NCCLS standards it is clearly understood that these organisms started resisting the existing antibiotics. It is mandatory to understand the resisting pattern to combat these resisting factors. Mradula Singh 2009 reported that the MIC levels of OFL showed 2-8 folds reduction in presence of CCCP (16/45; 35.5%), verapamil (24/45; 53.3%) and DNP (21/45; 46.6%) while in case of isolates identified as OFL sensitive these did not show any effect on ofloxacin MICs. In 11 of

45 (24.5%) isolates change in MIC levels was observed with all the three inhibitors. Overall 30 (66.6%) isolates had reduction in OFL MIC after treatment with these inhibitors. Also we need to understand the transforming ability of plasmids to species. This may be the most devastating reason to spread of resistance characters within the bacteria in and around a specific region. So in this study we decided and performed the transformation studies of resistance making plasmids isolated from clinical isolates resulting from sensitivity assay and MIC analysis.

The effectiveness of this modified resazurin assay has been demonstrated with methanol extracts of selected Scottish plants, purified compounds and the positive control ciprofloxacin, and a direct comparison of the MIC determination of the antibiotics using the old and modified resazurin methods using the antibiotics norfloxacin, cefotaxime, and amoxicillin.(Satyajit D 2007).

The result shown profound to accurate value on repeated assay .By this method seems to be very effective in determining the minimum inhibitory concentration of any compound or extract

Minimum inhibitory concentration

Photo 3

Photo 4

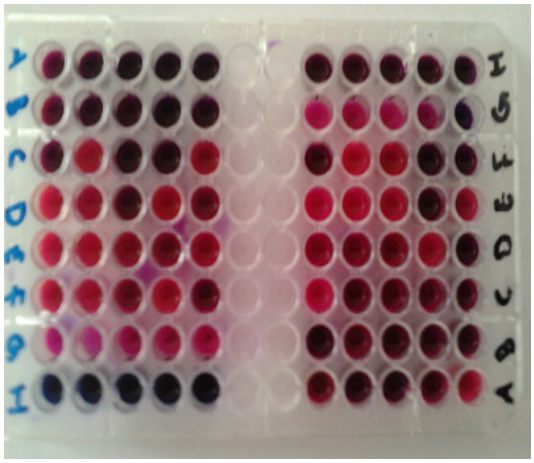


Photo 5

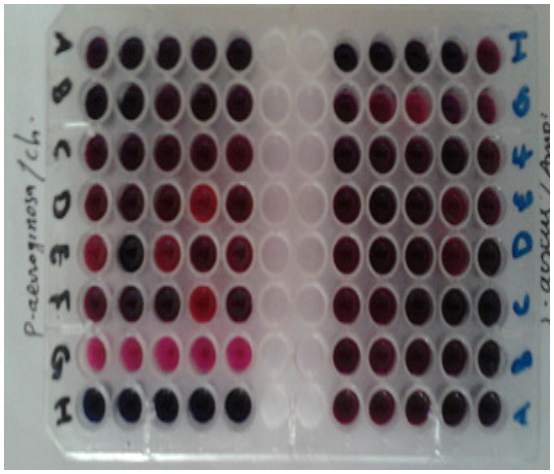


Photo 6

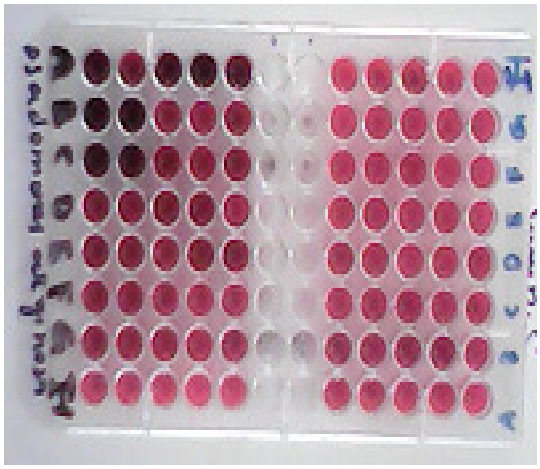


Photo 3- MIC of ceftriaxone (Right/ Black) and roxithromycin (Left/ Blue)
against *P. aeuroginosa*
Photo 4- MIC of ceftriaxone (Right/ Black) and roxithromycin (Left/ Blue)
against *S. aeureus*.
Photo 5- MIC of chloromphenicol (Left/ Black) against *P. aeuroginosa* and ampicillin (Right/Blue) against *S. aeureus*.
Photo 6- MIC of chloromphenicol (Left/ Black) against *S. aeureus*. and ampicillin (Right/Blue) against *P. aeuroginosa*.
A- 250µg/ml, B- 125 µg/ml, C- 62.5 µg/ml, D-31.25 µg/ml, E-

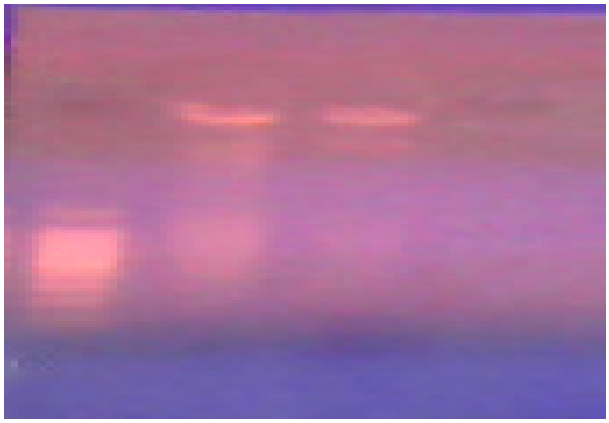
Plasmid DNA isolation

The charaterization of the plasmid isolated from resistant bacteria was studied by agarose gel electrophoresis.

From the photo 7, lane 1 is the marker DNA utilized having basepair size ranging from 10-100 bp. Lane 2 represents plasmid isolate from P.aeuroginosa and lane 3 depicts the plasmid isolated from S.aureus. the seperated plasmids showed high molecular comparing to the marker DNA utilized.

Photo 7: Agarose Gel Electrophoresis

Lane1 ↓ Lane2 ↓ Lane 3 ↓



Lane 1: Marker DNA(10-100bp)

Lane 2: Plasmid from *P.aeuroginosa*

Lane 3:Plasmid from *S.aureus*

To determined that, if the bacterial resistant to antibiotic is encoded by plasmid DNA, by transformation process was performed with plasmid isolated from *P.aeruginosa* and *S.aurus* and *E.coli DH5α* strain The isolated plasmid DNA from *P. aeruginosa* and *S.aurus* transferred successfully to *E. coli DH5α* strain.

Nizami Duran 2007 reported the Methicillin resistance rate among 139 Staphlococcus aureus isolates was 16.5 and 25.9 per cent of *S. aureus* carried mecA gene. Of the 159 CoNS isolates, methicillin resistance rate was 18.9 and 29.6 per cent carried mecA gene. Ninety four isolates identified as gentamicin resistant phenotypically, contained at least one of the gentamicin resistance genes [aac(6')/aph(2''), aph(3')-IIIa, ant(4')-Ia], 17 gentamicin-susceptible isolates were found as positive in terms of one or more resistance genes [aac(6')/aph(2''), aph(3')-IIIa, ant(4')-Ia] by multiplex PCR. A total of 165 isolates were resistant to erythromycin, and contained at least one of the erythromycin resistance genes (ermA, ermB, ermC and msrA). Phenotypically, 106 staphylococcal isolates were resistant to tetracycline, 121 isolates carried either tetK or tetM or both resistance genes. The majority of staphylococci tested possessed the blaZ gene (89.9%).

Adel KK 2010 determined if the antibiotic resistance in isolated *P. aeruginosa* is encoded by plasmid DNA or chromosomal DNA transformation process. They performed the transformation process to the most resistant isolate of *P. aeruginosa* P8 and more sensitive isolate P1 and E. coli DH5" strain. Extracted plasmid DNA from *P. aeruginosa* transferred successfully to E. coli DH5" strain. Ninety nine and thirty six transforment colonies obtained for P1 and P8 isolates, respectively.

Table 6: No of colonies seen in Transformed plates

S.No	Strains	Colonies
1	*Pseudomonas aeuroginosa* transformed plasmid to *DH5α*	26

2	*Staphylococcus aureus* transformed plasmid to *DH5* α	24

Photo 8

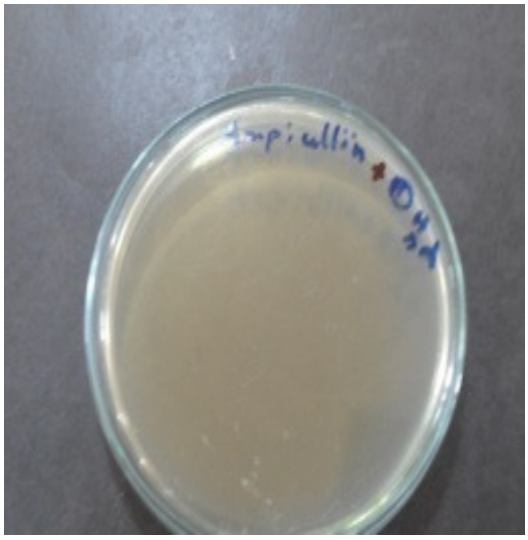


Photo 9

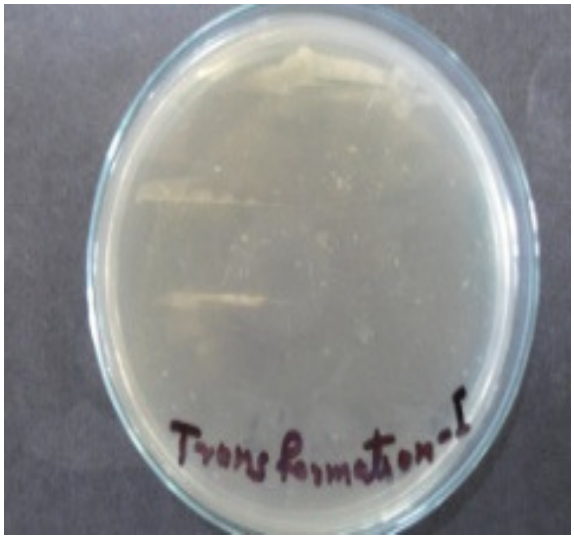


Photo 10

Photo 11

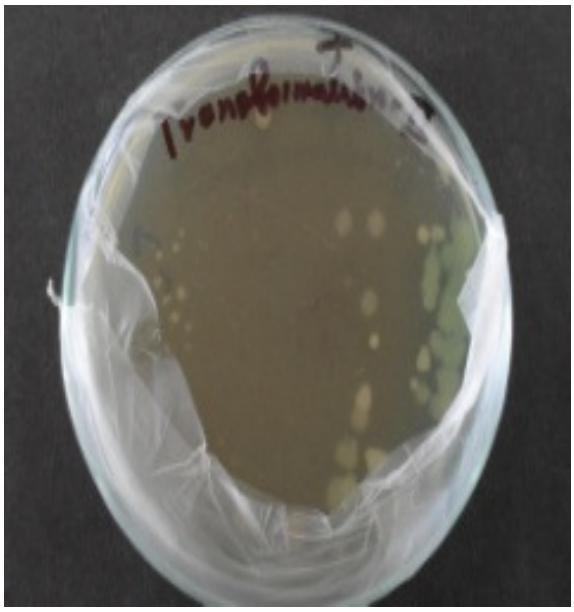


Photo 8- Control plate (Transformation I) contains DH5α with ampicillin
Photo 9- Transformation I- Competent cells from DH5 α with plasmid DNA isolated from *P.aeuroginosa* and ampicillin.
Photo 10- Control plate (Transformation II) contains DH5α with choloramphenicol
Photo 11- Transformation II- Competent cells from DH5 α with plasmid DNA isolated from *S.aeureus* choloramphenicol.

Efflux Pump Assay

Ethidium bromide Accumulation:

The existence of antibiotic efflux pumps was analyzed by ethidium bromide accumulation (1 µg•ml) in bacterial cells treated with test antibiotic by exploring the fluorescence of Ethidium bromide bacteria under UV light. There was an accumulation of Etidium bromide in antibiotic treated treated bacteria compared to control when examined under UV light (photos.12,13,14,15.)

Edward A,*et al* 2012 analysed and repoted the existence of solvent efflux pumps was analyzed by rhodamine B accumulation (100 µg•ml) in bacterial cells treated with test solvents by exploring the fluorescence of Rhodamine B in bacteria under UV light. Could observed clear an accumulation of Rhodamine B dye in organic solvents treated bacteria compared to control when examined under UV .from this study they reported that the frequent exposure of microbes to organic solvent may induce the resistance pattern of microbes toward antibiotic.

Gene deletion resulted in a drastic decrease in fluorescence, clearly indicating the active efflux of these drugs from hns cells Accumulation of these drugs in E. coli cells was observed from the fluorescence of ethidium bromide and rhodamine 6G under UV light. .(Kunihiko Nishino 2004)

Photo 12 **Photo 13**

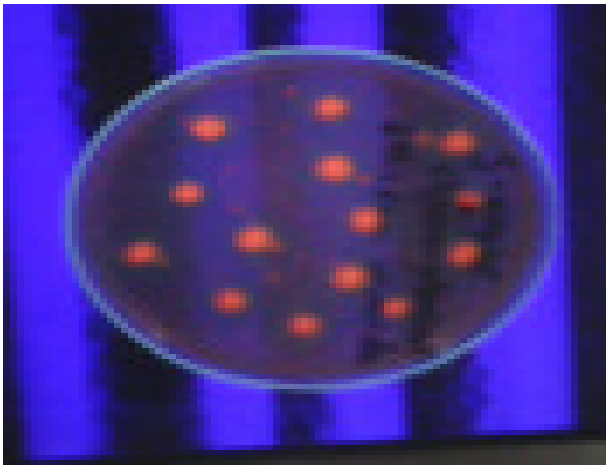
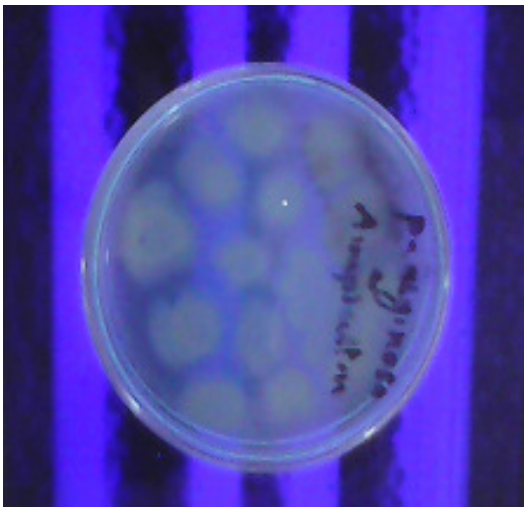


Photo 14 **Photo 15**



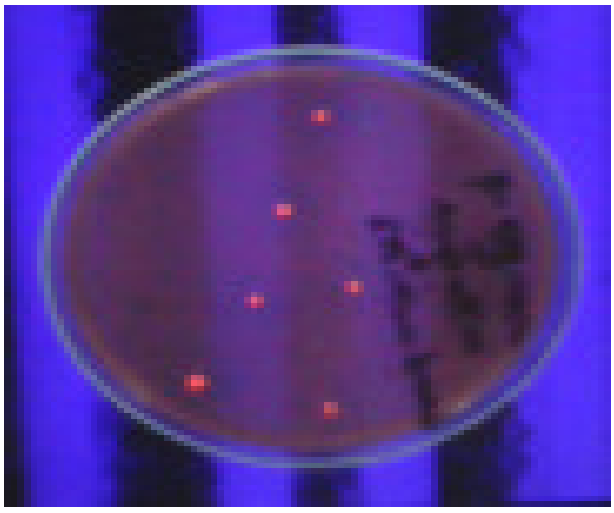


Photo 12- Control plate (*S.aeureus* with choloramphenicol) under UV transilluminator

Photo 13- Accumulation of ethidium bromide in S.aeureus existing with choloramphenicol under UV transilluminator.

Photo 14- Control plate (P.*aeuroginosa* with ampicillin) under UV transilluminator

Photo 15- Accumulation of ethidium bromide in P.*aeuroginosa* existing with ampicillin under UV transilluminator.

Ethidium bromide Accumulation Assay

The accumulation assay was performed to determine the activity of efflux pumps in the bacterial cells. Etidium bromide was utilize as a test dye for the accumulation assay. *P.aeruginosa* accumulation dye at 30 min and *S.aureus* accumulating dye at 15th min, after which there was a sudden decline in the accrual of dye till 45 min followed by gradual increase and decrease in the accumulation of dye .(table 7 and chart .1)

Ethidium bromide efflux assay

P.aerginosa and s.aurus has shown high efflux activity of Etidium bromide at 1000mM (glucose containing PBS) followed by 100, 50 and 10mM compared to control (glucose free PBS). *P.aerginosa* efflux activity was observed at 45th minute and for *S.aurus*. It was observed at 60th mn incubated with glucose has shown high efflux activity compared to untreated.

B. oleronius has shown high efflux activity of rhodamine B at 1000mM (glucose containing PBS) followed by 100, 50 and 10mM compared to control (glucose free PBS). B. oleronius incubated with glucose has shown high efflux activity compared to untreated. Efflux activity was observed at 45 min after the incubation(Edwar A, (2012). This above study coincides with our results upon the glucose concentrations.

Table 7: Ethidium bromide accumulation assay

S.No	Time Interval (min)	Optical density (600nm)	
		Staphylococcus aureus	***Pseudomonas aeuroginosa***
1	0	0.24	0.16
2	15	0.34	0.22
3	30	0.26	0.30
4	45	0.29	0.10
5	60	0.18	0.16
6	75	0.08	0.10
7	90	0.15	0.11
8	105	0.10	0.15
9	120	0.06	0.13
10	135	0.05	0.10

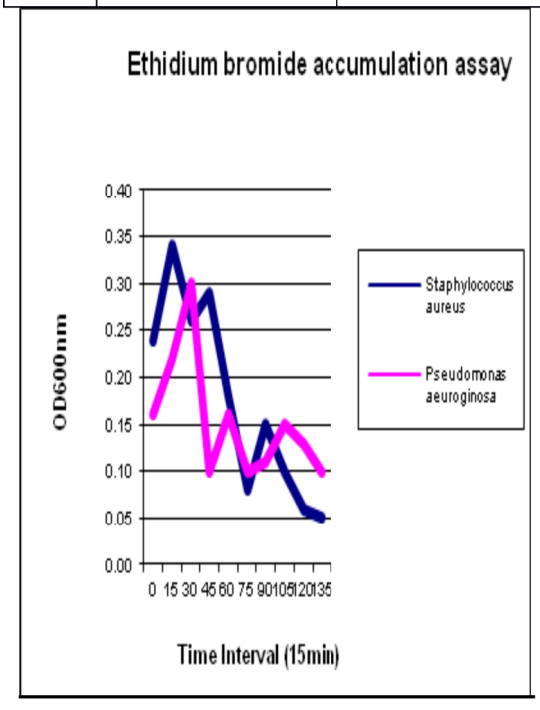


Chart No 1

Table 8: Influence of Glucose concentration in efflux pump assay

S. No	Time interval (Min)	Glucose Concentration (mM)							
		10		50		100		1000	
		P.aeuro	*S.aureus*	*P.aeuro*	*S.aureus*	*P.aeuro*	*S.aureus*	*P.aeuro*	*S.aureus*
1	0	0.34	0.25	0.17	0.10	0.22	0.15	0.25	0.20
2	15	0.11	0.18	0.18	0.13	0.30	0.20	0.20	0.23
3	30	0.19	0.15	0.22	0.12	0.17	0.17	0.25	0.18
4	45	0.26	0.11	0.25	0.10	0.16	0.22	0.45	o.15
5	60	0.12	0.13	0.19	0.11	0.13	0.09	0.23	0.38
6	75	0.10	0.08	0.12	0.12	0.18	0.16	0.16	0.27
7	90	0.08	0.05	0.11	0.07	0.12	0.10	0.14	0.13
8	105	0.09	0.10	0.20	0.10	0.18	0.08	0.19	0.22
9	120	0.06	0.13	0.20	0.15	0.15	0.21	0.10	0.10

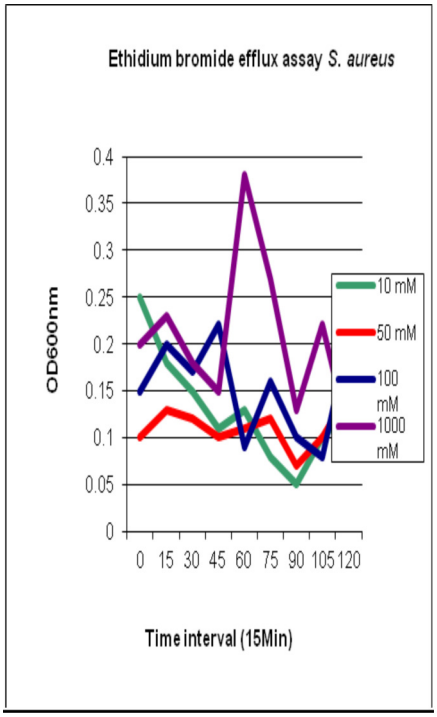
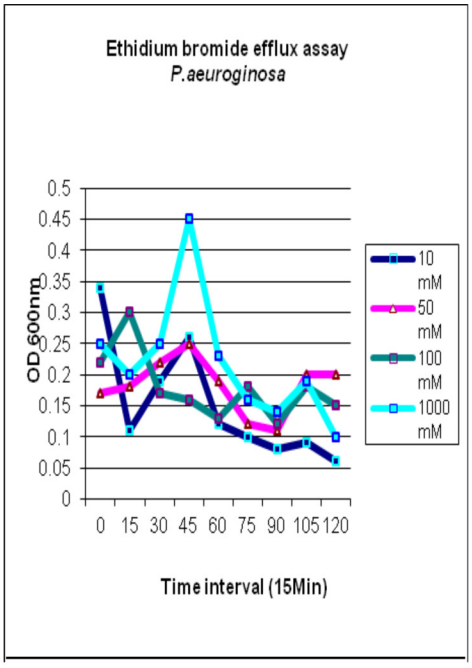

Ethidium bromide efflux assay S. aureus
OD600nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0 15 30 45 60 75 90 105 120
Time interval (15Min)
10 mM
50 mM
100 mM
1000 mM

Chart No.2


Ethidium bromide efflux assay
P.aeuroginosa
OD 600nm
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0 15 30 45 60 75 90 105 120
Time interval (15Min)
10 mM
50 mM
100 mM
1000 mM

Exposure to biocides and dyes are commonly employed in hospital and laboratory settings. Many of these agents are substrates for multiple-drug resistance (MDR)-conferring efflux pumps of both Gram-positive and Gram-negative organisms. Several such pumps have been identified in *Staphylococcus aureus*, and mutants overexpressing the NorA and MepA MDR pumps following exposure to fluoroquinolones have been identified. Such problem is seen in many of the case s related to resistant to antibiotic. The effect of exposure to low concentrations of biocides and dyes on the expression of specific pump genes has not been evaluated (Aure lie A. Huet, Jose L. Raygada, (2008)). in this study we determined the efflux activity of *staphylococcus* aureus and *Pseudomonas aeruginosa* as such and their increase ability with respect to glucose concentration.

This efflux active it may be due to over expression of gens which resist ampicillin and chloramphenicol.

7. SUMMARY AND CONCLUSIONS

Pseudomonas aeruginosa and Staphylococcus aureus is an opportunistic pathogen that causes human infection; and they showed high resistant to most antibiotic (ampicillin, chloromphenicol), Transformation process results derives us to conclude that all genes responsible for antibiotic resistant in *Pseudomonas aeruginosa* and *Staphylococcus aureus* from the findings of this study, This has been attributed to either the ability of these organisms to produce resistance mechanisms, for this the characterization of plasmid DNA of *P.aeruginosa* and *Staphylococcus aureus* isolates indicated that these two isolates transfer to another bacteria, in present study to *E. coli* DH5" strain.

Unfortunately, resistance in some species has developed to the level that no clinically available treatment is effective. Prevention and control strategies will require the application of epidemiological and behavioural approaches, as well as the research technologies aimed at the basic mechanisms of drug resistance.

The genetic characterization of antimicrobial resistance genes as well as their location and diversity is important in identifying factors involved in resistance. All the alternative strategies to overcome resistance require expanded knowledge of the molecular mechanisms of antibiotic resistance, their origins and evolution, and their distribution throughout bacterial populations and genomes.

Accumulation of Ethidium bromide was observed in effluxing of ethidium bromide was more in glucose treated cells than control. This has shown that the glucose has provided energy necessary to efflux ethidium bromide in and out of the cells.

Ethidium bromide accumulation and efflux activity in bacterial isolates divulged the critical importance of efflux pump in the extrusion of harmful compounds for the survival of bacteria under stressful environment.

Susceptibility analysis showed the *Staphyloccocus aureus* resistant to chloramphenicol and *Pseudomonas aeruginosa* to ampicillin.

The Minimum Inhibitory Concentration range between 7.81to 62.25 for ampicillin, chloramphenicol, roxithromycin and ceftriaxone tested against

Staphyloccocus aureus, whereas MIC range between 7.81 to 250 for chloramohenicol,ceftriaxone, roxithromycin and ampicillin tested against *Pseudomonas aeruginosa* .

The successful transformation was done and 26 colones where seen in transformed plate of *Pseudomonas aeruginosa* and 24 colones in transformed plates of *Staphyloccocus aureus.*

The accumulation assay and the influence of glucose concentration in effluxing ability where studied and optimum glucose concentration which influence efflux ability was found was found at 1000 Mm, by this we conclude the clinical isolates *Pseudomonas aeruginosa* and *Staphyloccocus aureus* resistant antibiotic ampicillin and chloramphenicol respectively through two different mechanism and genes present in plasmid. Either new drug moiety or modifications needed in existing antibiotics.

8. BIBLIOGRAPHY

1. Adel K.K. and Sabiha S.S. (2010). Genetic Site Determination of Antibiotic Resistance Genes in *Pseudomonas aeruginosa* by Genetic Transformation, British Journal of Pharmacology and Toxicology 1(2): 85-89

2. Adenike A. O. Ogunshe (2006) *in vitro* phenotypic antibiotic resistance in bacterial flora of some indigenous orally consumed herbal medications in Nigeria. Journal of Rural and Tropical Public Health 5: 9-15,

3. Alasdair P. MacGowan and Karen E. Bowker (2003). Mechanism of Fluoroquinolone Resistance Is an Important Factor in Determining the Antimicrobial Effect of Gemifloxacin against Streptococcus pneumoniae in an In Vitro Pharmacokinetic Model, Antimicrobial Agents and Chemotherapy, Mar. 2003, p. 1096–1100

4. Ameenas.Juma.M (2010). Genetic Transformation in antibiotic resistant *escherichia Coli* O. Medical Journal of Islamic World Academy of Sciences 18:2, 75-84, 2010

5. Amin *et al* (2009). Prevalence of antibiotic resistance among clinical isolates of *Klebsiella pneumonia* isolated from a Tertiary Care Hospital in Pakistan. Malaysian Journal of Microbiology, Vol 5(2) 2009, pp. 81-86

6. Anandi Martin, Mirtha Camacho, Franc¸ oise Portaels,1 and Juan Carlos Palomino (2003). Resazurin Microtiter Assay Plate Testing of Mycobacterium tuberculosis Susceptibilities to Second-Line Drugs: Rapid, Simple, and Inexpensive Method,, Antimicrobial Agents and Chemotherapy, Nov. 2003, p. 3616–3619

7. Anja S. Hummel, Christian Hertel, (2007). Stated the antibiotic Resistances of Starter and Probiotic Strains of Lactic Acid Bacteria. Applied and Environmental Microbiology, Feb. 2007, p. 730–739

8. Ashraf, R. and Shah, N. P (2011). Antibiotic resistance of probiotic organisms and safetyof probiotic dairy products., International Food Research Journal 18(3): 837-853

9. Asma bashir Talat Y. Mujahid and Nayar Jehan (2007). antibiotic resistance profile: isolation And characterization of clinical isolates of staphylococci from

Patients with community acquired skin infections. Pak. J. Pharm. Sci., 2007, Vol.20(4), 295-299

10. Aure lie A. Huet, Jose L. Raygada, (2008). Multidrug efflux pump over expression in *Staphylococcus aureus* after single and multiple in vitro exposures to biocides and dyes. Microbiology (2008), 154, 3144–3153
11. David Dubnau (1999). DNA uptake in bacteria. Annu. Rev. Microbiol. 1999. 53:217–44
12. Desj Simeonia, Lucia Rizzotti *et al* (2007). Study Antibiotic resistance genes and identification of staphylococci collectedfrom the production chain of swine meat commodities. Food Microbiology 25 (2008) 196–201
13. Didier hocquet *etal* 2007. *pseudomonas aeruginosa* may accumulate drug resistance mechanisms without losing its ability to cause bloodstream infections. antimicrobial agents and chemotherapy, oct. 2007, p. 3531–3536
14. Douglas Hanahan (1983). Transformation of *Escherichia coli* with Plamid.J.Mol.biol 166,557,580
15. Edward A *et al* (2012). Role of Efflux Pumps in Affording Organic Solvent Tolerance to Bacterial Strains from Petroleum Contaminated Soil, Middle-East Journal of Scientific Research 12 (1): 80-87, 2012 ISSN 1990-9233
16. Fred C. Tenover (2006). Mechanisms of Antimicrobial Resistance in Bacteria, the American Journal of Medicine (2006) Vol 119 (6A), S3–S10
17. Gary S. Gray (1980) Evolution of Antibiotic Resistance Genes: the DNASequence of a Kanamycin Resistance Gene from s*taphylococcus Aureus*.
18. Glenn W. Kaatz (2000) evidence for the Existence of a Multidrug Efflux Transporter Distinct from Nor A in *Staphylococcus aureus.* Antimicrobial Agents and Chemotherapy, p. 1404–1406
19. Gulcan ozbakir1, Hande avcioglu and isil seyis bilkay 2010. Determination of antibiotic resistance and plasmid dna profiles of *esherichia coli* strains isolated from clinical materials. international journal of agriculture & biology print: 1560–8530
20. Hiroshi Nikaido (2009). Multidrug Resistance in Bacteria. Annu Rev Biochem. 2009; 78: 119–146. doi:10.1146 / annurev.biochem.78. 082907.145923

21. Hleba L., Kacaniova M, Pochop,j, Lejkova,j., Cubon, Kunova,s 2011. antibiotic resistance of *enterobacteriaceae* genera and *salmonella* spp., *salmonella enterica* ser. typhimurium and enteritidis isolated from milk, cheese and other dairy products from conventional farm in Slovakia,. journal of microbiology, biotechnology and food science.

22. Janne Kataja, Pentti Huovinen, Mikael Skurnik (1999). Streptococci in Finland erythromycin Resistance Genes in Group A,. Antimicrobial agents and chemotherapy, 0066-4804/99/$04.0010 Jan. 1999, p. 48–52

23. Keith Poole 2001, Multidrug Efflux Pumps and Antimicrobial Resistance in pseudomonas aeruginosa and Related Organisms, J. Mol. Microbiol. Biotechnol. 3(2): 255-264

24. Kunihiko Nishino and Akihito Yamaguchi (2004). Role of Histone-Like Protein H-NS in Multidrug Resistance ofEscherichia coli. Journal of Bacteriology, Mar. 2004, p. 1423–1429

25. Lateef.A J. K. Oloke and E. B. Gueguimkana (2003). The prevalence of bacterial resistance in clinical, food, water and some environmental samples in Southwest Nigeria. Environmental Monitoring and Assessment (2005) 100: 59–69

26. Laura Paixao (2009). Fluorometric determination of ethidium bromide efflux kinetics in *Escherichia coli*. Journal of Biological Engineering

27. Lazoraie M,M (2009). Mechanisms Involved In Organic Solvent Resistance in Gram-Negative Bacteria World Academy of Science, Engineering and Technology 30 2009

28. Magdalena Chroma, Milan Kola (2010) Genetic Methods for Detection of Antibiotic Resistance: Focus on Extended-Spectrum B-Lactamase. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2010; 154:XX

29. Mahipal Singh, Arpita Yadav, Xiaoling Ma and Eugene Amoah (2010) Plasmid DNA Transformation in *Escherichia Coli:* Effect of Heat Shock Temperature, Duration, and Cold Incubation of $CaCl_2$ Treated Cells. International Journal of Biotechnology and Biochemistry. ISSN 0973-2691 Volume 6 Number 4 (2010) pp. 561–568

30. Manju Bala, Krishna Ray & S.M. Gupta (2005). Comparison of disc diffusion results with minimum inhibitory concentration (MIC) values for antimicrobial susceptibility testing of *Neisseria gonorrhoeae*. Indian J Med Res 122, July 2005, pp 48-51

31. Melanie P. Dautle *et al* (2004) In Vitro Antibiotic Sensitivity and Resistance of 100 Clinical Bacterial Isolates Purified from Microbial Bio films Associated with Silicone Gastrostomy Tubes Removed from Pediatric Patients. The Journal of Applied Research Vol. 4, No. 1, 2004

32. Mradula Singh, G.P.S. Jadaun *et al* (2011). Effect of efflux pump inhibitors on drug susceptibility of ofloxacin resistant Mycobacterium tuberculosis isolates,. Indian J Med Res 133, May 2011, pp 535-540

33. Mustafa Oskay, Dilek Oskay and Fatih Kalyoncu. (2009). The activity of Some Plant Extracts Against Multi-Drug Resistant Human Pathogens. Iranian Journal of Pharmaceutical Research (2009), 8 (4): 293-300

34. Neal.C, Stewart (1996). Genetic Transformation, Recovery, and Characterization ofFertile Soybean Transgenic for a Synthetic *Bacillus fhuringiensis* cry/Ac Gene, Plant Physiol. (1996) 112: 121-129

35. Neetu Kumra Taneja and Jaya Sivaswami Tyagi (2007). Resazurin reduction assays for screening of anti-tubercular compounds against dormant and actively growing Mycobacterium tuberculosis, Mycobacterium bovis BCG and Mycobacterium smegmatis. Journal of Antimicrobial Chemotherapy (2007) 60, 288–293

36. Niimi. K., Maki. K. (2005). Over expression of Candida albicans CDR1, CDR2, or MDR1 Does Not Produce Significant Changes in Echinocandin Susceptibility. Antimicrobial Agents and Chemotherapy, Apr. 2006, p. 1148–1155

37. Nizami Duran, Burcin Ozer, Gulay Gulbol Duran, Yusuf Onlen & Cemil Demir (2012). Antibiotic resistance genes & susceptibility patterns in staphylococci,. Indian J Med Res 135, March 2012, pp 389-396

38. Notani N.K, V. P. Joshi. V. P. and. Kanade R. P (1984) Genetic transformation in bacteria. *J. Biosci.*, Vol. 6, Number 4, October 1984, pp. 525–533

39. Olusegun O. Soge, Bolanle A. Adeniyi1 and Marilyn C. Roberts (2006). New antibiotic resistance genes associated with CTX-M plasmids from uropathogenic Nigerian Klebsiella pneumonia. Journal of Antimicrobial Chemotherapy (2006) 58, 1048–1053

40. Richard C. Honour (2003), Antibiotic-Resistant Microbes: Global Superbugs; Global Killers

41. Ricki lewis, (1995). The rise of antibiotic resistant infection FDA consumer magazine

42. Roberto Cabrera, Joaquím Ruiz, *et al* (2004). Mechanism of Resistance to Several Antimicrobial Agents in Salmonella Clinical Isolates Causing Traveler's Diarrhea, Antimicrob Agents Chemother. 2004 October; 48(10): 3934–3939.

43. Satyajit D. Sarker, Lutfun Nahar, and Yashodharan Kumarasamy (2007)Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals

44. Senka Didi, Jagoda (2007). Antibiotic Resistance Mechanisms in Bacteria: Biochemical and Genetic Aspects. Antibiotic Resistance in Bacteria, *Food Technol. Biotechnol. 46* (1) 11–21

45. Sharon D. Cosloy and Michio Oishi (1972). Genetic Transformation in *Escherichia coli* K12. Proc. Nat. Acad. Sci. USA Vol. 70, No. 1, pp. 84-87, January 1973

46. Simeon Oloni Kotchoni et al (2003) A home made kit for plasmid DNA mini-preparation. African Journal of Biotechnology Vol. 2 (4), pp. 88–90,

47. Srisurang Tantimavanich Churairatana Nilakul and Samaniya Sukroongreun (*2002).*Modified Antimicrobial Disc Susceptibility Testing for Nutritionally - Variant Streptococci. Southeast Asian J Trop med public health

48. Tanya Strateva and Daniel Yordanov 2009. Pseudomonas aeruginosa phenomenon of bacterial resistance. Journal of Medical Microbiology (2009), 58, 1133–1148.

49. Thomas P. Quinn and Katrina T (1997). Trevor Isolation and Quantification of Episomal Expression Vectors in Human Tcells. Bio Techniques 23:1044-1046

50. Thomas Schwartz a, Wolfgang Kohnen b, Bernd Jansen b, Ursula Obst (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water bio ¢ lms, FEMS Microbiology Ecology 43 (2003) 325-335

51. Webber M.A. and L. J. V. Piddock (2003). The importance of efflux pumps in bacterial antibiotic resistance. *Journal of Antimicrobial Chemotherapy* (2003) 51, 9–11

52. Worldying Zhang Baltimore (2007). Mechanisms of Antibiotic Resistancein the Microbial USA.